Rhizofiltration of urban effluent: microbial ecology and conceptual treatment mechanisms

By

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Dissertation presented for the degree of
Doctor of Microbiology in the
Faculty of Science at Stellenbosch University

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March 2016

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2016

Summary

Polluted urban runoff is a challenge that is globally met by governing bodies employing best management practices (BMPs). One such BMP is rhizofiltration, a novel type of phytoremediation BMP designed to mimic riparian ecology, with the goal of rapidly filtering large volumes of urban runoff before it enters rivers. The physical, chemical and biological mechanisms behind pollutant removal within a rhizofiltration system however, are still largely unknown. The overall aim of this study was therefore to assess the ability of a pilot scale rhizofiltration system to reduce concentrations of the physico-chemical pollutants ammonium, chemical oxygen demand (COD), nitrate, phosphate, sulphate and suspended solids, as well as microbial indicators of faecal pollution, in simulated urban runoff. The faecal indicators included coliphages, faecal coliforms, potentially pathogenic yeasts (PPY) and *Salmonella* and/or *Shigella.* To achieve this study's aim a conceptual model was first constructed to identify potential bacterial mechanisms of pollution removal and to estimate the effect of physico-chemical conditions on microbial communities within the rhizofilter medium. Then, the overall performance of the filter was measured with regard to its bioregeneration and sorption capacity for the abovementioned pollutants. Sorption equilibrium, for most of the pollutants in the simulated runoff percolating through the filter, was reached within 45 minutes. Partial bioregeneration of the filter medium occurred within a week for ammonium, COD, phosphate and sulphate, as well as for the microbial pollutants. Evidence was subsequently obtained supporting the hypothesis that this regeneration is brought about by microbial activity, since metagenomic (16S rDNA high throughput sequencing) and phospholipid fatty acid (PLFA) analyses revealed the presence of viable dynamic microbial populations within the rhizofilter medium. Significant correlations between

relative quantities of microbial operational taxonomic units (OTUs) and nutrient concentrations were also uncovered. The rhizofilter plants selected for a microbial community distinct from an unplanted control, however, this did not relate to differences in filter performance. This phenomenon was ascribed to the rapid percolation rate and design of the rhizofilter which maximizes aeration of the filter medium. It was contended that these properties, combined with the composition of the simulated urban runoff, selected for functionally similar organisms. The Actinomycetales were the most abundant bacterial group in both the planted and unplanted filter media. However, the plants appeared to select for Mycobacteriaceae and nitrifiers identified as the Nitrospiraceae. Among the transient OTUs in the filter media were taxa associated with the human gut, including the Campylobacteraceae, Moraxellaceae, Porphyromonadaceae and Prevotellaceae, while the Enterobacteriaceae containing faecal coliforms were below the detection limit of the metagenomic analysis. Strains of four *Candida* species consistently occurred in the simulated urban runoff. The abundance of these PPY in the influent and effluent of the rhizofilter were affected by physico-chemical factors. Subsequent metagenomic analysis of the fungal community within the filter media revealed a low relative abundance of candidal PPY. In short, the rhizofilter design and wastewater composition selected for copiotrophic aerobic microorganisms capable of mineralizing potentially recalcitrant organic carbon and driving oxidative processes such as nitrification whilst removing human microbial commensialists and pathogens.

Opsomming

Besoedelde stedelike afloop is ’n uitdaging wat wêreldwyd deur bestuursliggame aangespreek word deur beste bestuurspraktyke (BBP). Een so ’n BBP is risofiltrasie, ’n nuwe tipe fitoremediëring BBP wat ontwerp is om oewerekologie na te boots, met die doel om groot volumes stedelike afloopwater vinnig te filtreer voor dit riviere bereik. Die fisiese, chemiese en biologiese meganismes wat die grondslag van risofiltrasie vorm, is egter nog grootliks onbekend. Die oorhoofse doel van hierdie studie was daarom om die vermoë van ‘n proefskaal risofiltrasiesisteem te toets vir die vermindering van konsentrasies van die fisikochemiese besoedelstowwe insluitende ammonium, chemiese suurstofvraag (CSV), nitraat, fosfaat, sulfaat en gesuspendeerde vaste stowwe, asook mikrobiese indikators van fekale besoedeling, in gesimuleerde stedelike afloop. Die fekale indikators sluit in kolifage, fekale koliforme, potensiële patogeniese giste (PPG) en *Salmonella* en/of *Shigella.* Om hierdie studie se doelwit te bereik, is ‘n konseptuele model gemaak om potensiële bakteriese meganismes van besoedelingverwydering te identifiseer en om die effek van fisikochemiese kondisies op mikrobiese gemeenskappe in die risofiltermedium te beraam. Daarna is die algehele werkverrigting van die filter gemeet ten opsigte van sy bioregenerasie en sorpsiekapasiteit van die bogenoemde besoedelstowwe. Sorpsie-ekwilibrium vir die meeste van die besoedelstowwe in die gesimuleerde afloop wat deur die filter geperkuleer het, is binne 45 minute bereik. Gedeeltelike bioregenerasie van die filtermedium het binne ‘n week plaasgevind vir ammonium, CSV, fosfaat en sulfaat, asook vir die mikrobiese besoedelstowwe. Bewyse is gevolglik gevind wat die hipotese ondersteun dat hierdie regenerasie aangewakker is deur mikrobiese aktiwiteit, aangesien metagenomiese (16S rDNA hoë omset volgordebepaling) en fosfolipied-vetsuuranalises (FLVS) die teenwoordigheid van

lewensvatbare dinamiese mikrobiese populasies in die risofiltermedium aan die lig gebring het. Beduidende korrelasies tussen relatiewe hoeveelhede van mikrobiese operasionele taksonomiese eenhede (MOTE) en nutriëntkonsentrasies is ook blootgelê. Die risofilter het vir ‘n mikrobiese gemeenskap geselekteer wat verskil van die ongeplante kontrole, hoewel dit nie aanleiding tot verskille in filterwerking gegee het nie. Hierdie verskynsel is toegeskryf aan die vinnige perkuleringstempo en ontwerp van die risofilter wat belugting van die filtermedium optimaliseer. Dit is geargumenteer dat hierdie eienskappe, saam met die samestelling van die gesimuleerde stedelike afloop, vir funksioneel eenderse organismes selekteer. Die Actinomycetales was die algemeenste bakteriese groep in beide die geplante en ongeplante filtermedia. Dit het egter voorgekom of die plante selekteer vir Mycobacteriaceae en nitrifiseerders wat as die Nitrospiraceae geïdentifiseer is. Onder die kortstondige MOTE in die filtermedia was taksa wat geassosieer word met die menslike dermkanaal, soos die Campylobacteraceae, Moraxellaceae, Porphyromonadaceae en Prevotellaceae, terwyl die Enterobacteriaceae-bevattende fekale koliforme laer as die waarnemingsvlak van die metagenomiese analise was. Stamme van vier *Candida* spesies het gereeld in die gesimuleerde stedelike afloop voorgekom. Die talrykheid van hierdie PPG in die invloei en uitvloei van die risofilter is deur fisikochemiese faktore beïnvloed. Daaropvolgende metagenomiese analise van die fungusgemeenskap in die filtermedia het daarop gedui dat *Candida* spesies 'n lae relatiewe voorkoms gehad het. Kortom, die risofilter en afvalwatersamestelling selekteer vir kopiotrofiese aerobe mikroörganismes wat in staat is om potensieel moeilik afbreekbare organiese koolstof te mineraliseer en oksidatiewe prosesse soos nitrifikasie te dryf, terwyl dit menslike kommensialiste en patogene verwyder.

Motivation

The availability of safe drinking water is a basic human right and essential for health (Hodgson and Manus, 2006). However, the world faces a number of problems due to inadequate access to, and ineffective management of, water resources (Gleick, 1998). Water scarcity has quickly spread to many regions of the world due to increases in population size, as well as consumption levels (Postel, 2000). In addition, pollution from various sources reduces the quantity of potable water. Urban runoff was identified as a major contributor of pollution in aquatic ecosystems (Fletcher et al., 2008; Göbel et al., 2007; Hathaway and Hunt, 2010). Many factors, including land use and the development status of a country, determine the composition and severity of polluted urban runoff (Walsh, 2000). Nutrients and anthropogenic chemicals in urban runoff may cause eutrophication and other detrimental ecological effects (De-Bashan and Bashan, 2004; Oberholster et al., 2008). In developing countries the dissemination of pathogens through polluted urban runoff is often of greater concern. Globally there are 46 countries where less than half the population, which amounts to 2.5 billion people, have access to sanitation facilities (World Health Organization and UNICEF, 2014). Consequently, in these countries there is a significant exposure risk to waterborne pathogens causing gastroenteritis, such as bacteria belonging to the genera *Salmonella* and *Shigella* (Levantesi et al., 2012; Mansilha et al., 2010). Moreover, immunocompromised individuals may be at greater risk in contracting infections by opportunistic fungal pathogens, such as yeasts belonging to the genus *Candida* that are known to survive in sewage polluted waters (Brinkman et al., 2003; Efstratiou and Tsirtsis, 2009; Woollett and Hedrick, 1970).

A need was therefore identified for engineered systems that can augment, diffuse, and point sources of urban runoff, and protects ecosystems as well as people living in polluted environments (Granato, 2014; Jia et al., 2015). These systems, referred to as Best Management Practices (BMPs), are often designed based on “black box” models and empirical performance data (Tietz et al., 2008). Therefore, little is known about the mechanisms of pollutant removal which are hypothesized to be mediated primarily by microorganisms (Faulwetter et al., 2009). Recently, a BMP incorporating rhizofiltration as a pollutant treatment process in an aerobic sand and rock filter medium bed was evaluated as a possible urban runoff treatment option (Wilsenach et al., 2014). The aim of this thesis was to use a pilot scale system of the aforementioned rhizofilter to contribute knowledge on microbial pollutant treatment mechanisms that may occur in this and similar BMPs.

With the above as background the first objective of this study was to create a conceptual model on treatment processes in the rhizofilter and the factors that may affect them (Chapter 1). The second objective was to test the bio-regeneration and sorption capacity of the rhizofilter to obtain an indication of the amount and rate at which pollutants can be removed from urban effluent (Chapter 2). The third objective was to determine the dynamics and composition of the active bacterial community in the rhizofilter media, as well as the effect plants may have in selecting specific bacterial populations (Chapter 3). The survival of enteric bacteria in the filter media was also studied (Chapter 3). The fourth objective (Chapter 4) was to determine whether opportunistic pathogenic yeasts, able to grow at 37 °C, could survive in the filter media. Part of this objective was to determine their relative abundance

compared to other fungi, and how they are affected by changes in physico-chemical conditions within the filter medium (Chapter 4).

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“We give you thanks, O Lord God Almighty, who is, and was, and is to come;
because you have taken to you your great power, and have reigned.”

The Book of Revelation 11:17
King James Bible

Dedicated to my wife and parents.

Acknowledgements

I would like to express my sincere appreciation and gratitude to:

- My supervisor, Prof. Alfred Botha, for his invaluable guidance, support, encouragement and for creating a stimulating research environment.
- My co-supervisor, Dr. Marnel Mouton, for her constructive criticism which greatly improved the quality of this work.
- My wife, Anneke Postma, for her unwavering support, additional research ideas and help in finalizing this thesis.
- The Water Research Commission (WRC), East Rand Water (ERWAT) and National Research Foundation (NRF) for funding.
- Prof. Gideon Wolfaardt for procuring funding from ERWAT, and his valuable contributions to Chapter 3.
- Dr. Etienne Slabbert for the next generation sequencing as well as his help and advice in preparing Chapter 3 for publication.
- Everyone in the Botha lab for their support and for creating a pleasant working environment.
- The personnel and students at the Department of Microbiology.
- My family and friends, for their interest, motivation and support.

Table of Contents

Declaration i
Summary ii
Opsomming iv
Motivation vi
Acknowledgements xi
List of figures xvi
List of tables xxii
Abbreviations xxiii
Equations xxv
Total effluent volume (eq. 1) xxv
Quantity adsorbed (eq. 2) xxv
Total pollutant load (eq. 3) xxv
Percentage removal (eq. 4) xxv
Chapter 1 1
Microbial processes in a rhizofilter treating polluted urban runoff: A review and conceptual model 1
1 Introduction and background 2
2 Conceptual model development 5
3 Biosystems engineering of riparian zones 6
4 Bacterial populations in BMPs 7
4.1 Distribution of bacteria in BMP filter media 8
4.2 Biofilm processes 9
4.3 Effect of plants 11
5 Factors that influence biofilter microbial communities and performance 12
5.1 Nutrients in urban effluent 12
5.2 Redox potential 13
5.3 Mode of operation 14
5.4 Influence of seasons 15
6 Nutrient cycling 16
6.1 Organic carbon 16

6.2 Nitrogen 18
6.3 Phosphate 20
6.4 Sulphate 21
7 Treatment of bacterial pathogens 22
8 Conclusion 23
9 References 25
Figures 32
Chapter 2 35
The regeneration and sorption capacity of a pilot scale rhizofilter 35
1 Introduction 36
2 Materials and methods 37
2.1 Experimental setup 37
2.2 Biological regeneration / performance tests 37
2.3 Sorption capacity 39
2.4 Sorption capacity calculations 39
2.5 Statistical analysis 41
3 Results and discussion 41
3.1 Biological regeneration 41
3.2 Sorption capacity 44
4 Conclusions 49
5 References 50
Figures and tables 54
Chapter 3 67
Dynamics of a simulated riparian microbiome treating severely polluted urban effluent 67
1 Introduction 68
2 Materials and methods 70
2.1 Experimental design 70
2.2 Sampling 71
2.3 Chemical and microbiological analyses of water and sand 72
2.4 PLFA analysis and DNA extraction 72
2.5 High throughput sequencing and sequence quality control 73
2.6 Statistical analysis 73

2.7 Correlation and network analysis 74
3 Results 74
3.1 Bacterial community relative activity and diversity 74
3.2 Interaction between chemical factors and microbial communities 76
3.3 Taxonomic composition 76
3.4 Bacterial community interactions 78
3.5 Short term changes in microbial activity and community composition 80
4 Discussion 81
5 References 85
Figures 91
Chapter 4 98
Occurrence of opportunistic pathogenic yeasts in wastewater and their removal by rhizofiltration 98
1 Introduction 99
2 Materials and methods 102
2.1 Experimental setup 102
2.2 Yeast isolation and enumeration 102
2.3 Yeast identification 103
2.4 Physico-chemical analyses 103
2.5 Filter medium sampling 104
2.6 Fungal metagenome sequencing and sequence quality control 105
2.7 Statistical analysis 106
3 Results 107
3.1 Yeast Removal 107
3.2 Factors affecting PPY concentration and removal 108
3.3 Potentially pathogenic yeast communities 110
3.4 Factors affecting PPY species abundance 111
3.5 Fungal community in filter media 112
4 Discussion 113
5 Conclusions 117
6 References 119
Figures and tables 124

Chapter 5 135
1 General conclusions and future research 136
2 References 144
Figure 146
Supplementary material 148
Chapter 3 148
1 PLFA extraction 149
2 Modified primers 150
3 PCR conditions 150
4 Sequence analysis 150
Supplementary material 151
Chapter 3: Figures and tables 151
Supplementary material 159
Chapter 4 159
1 Yeast identification 160
2 Barcoded primers and DNA library preparation for high throughput sequencing 161
3 References 162
Supplementary material 163
Chapter 4: Figures and tables 163

List of figures

Chapter 1

Figure 1.1: The rhizofilter experimental setup. Settled municipal wastewater was pumped from a trickling filter distribution sump into the 10 000 L tank where it settled for a further 24 hours. Excessive solids were removed by flushing 2 000 L of the water prior to releasing the rest of the formulated influent (8 000 L) onto the planted and unplanted sides (insert) of the system at a rate of 7 $L.s^{-1}$. The water percolated vertically through the filter media for 45 minutes. 33

Figure 1.2: Conceptual model of treatment processes which may occur in the rhizofiltration system. (a) Urban effluent contains numerous pollutants but the focus of this study is on selected nutrients and microbial indicator organisms. Pollutant removal is hypothesized to be mediated by microbial biofilms (b) on surfaces such as the sand media (c) and plant roots (d) within the predominantly aerobic Rhizofilter matrix. Biofilm activity is a function of biofilm thickness and diversity which is influenced by the redox potential (Eh), physical properties (e) of the system and plant root exudates including oxygen (d). Organic carbon from the urban effluent (COD) and released by the plant roots (d) promotes the growth of heterotrophs (f) that may be aerobic and/or facultative anaerobic. Respiration by the heterotrophs produces CO_2 that is used as an inorganic carbon source by chemolithoautotrophs (g) which are responsible for nitrogen and sulphur cycles in the system (h). The products of the nutrient cycles could also influence biofilm characteristics. The biomass ratio between these two groups of bacteria is a function of their respective temperature dependant growth rates. Redox gradients in biofilms (c) and the rhizosphere (d) exist due to microbial respiration and differential spatial and temporal release of oxygen by the plant roots. This may allow for both aerobic and (restricted) anaerobic pollutant removal processes. All bacterial pathogens are heterotrophs (f) and thus, at some stage, form part of the heterotroph community in the rhizosphere. Studying the dynamics within the heterotrophic community could lead to important insights in pathogen removal mechanisms. 34

Chapter 2

Figure 2.1: Ammonium concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week..... 55

Figure 2.2: Nitrate concentrations and removal on the planted (R; experiement) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week..... 56

Figure 2.3: Chemical oxygen demand concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week. 57

Figure 2.4: Phosphate concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week..... 58

Figure 2.5: Sulphate concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week..... 59

Figure 2.6: Suspended solids concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week. 60

Figure 2.7: Faecal coliform concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week. 61

Figure 2.8: *Salmonella/Shigella* concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week. 62

Figure 2.9: Coliphage concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week..... 63

Figure 2.10: Breakthrough curves of the unplanted and planted sides of the rhizofilter with regards to the measured chemical and microbiological pollutants. Y-axis values are the effluent/influent ratios at 3 minute time intervals for a total duration of 48 minutes calculated from triplicate samples collected on three separate occassions. Curves are LOESS smoothed regressions and the shaded areas indicate a 95 % confidence interval. 64

Chapter 3

Figure 3.1: Boxplots of OTU diversity and richness of the three sand fractions, calculated using the Shannon diversity index and Chao richness estimate respectively. C=Control sand, PB= planted bulk sand, PR=rhizosphere sand. Symbols (D) indicate groups that were not significantly different (adjusted pairwise t test p > 0.05). 92

Figure 3.2: Nonmetric multi-dimensional scaling bi-plots showing the relative differences in OTU community composition. The Bray-Curtis distance metric was used to quantify the similarity between the observed patterns. Arrows (fitted vectors) indicate significance and direction of sand property correlations with the ordinations. Total carbon (C), carbon/ammonium ratio (C:NH_4), Chao richness, faecal coliforms (FC), nitrate (NO_3^-), phosphorus (P), Shannon diversity, and sulphur (S). PLFA - chain length:number of double bonds; Me = methyl side chains. Arrow length is proportional to the strength of the correlation (r). The significance of fitted vectors was determined using permutation tests (n=1000) at the $p<0.05$ significance level. Ellipses indicate a 95% confidence interval for replicates. The addition of a third dimension did not lead to a significant increase in the explanatory power of the ordinations..... 93

Figure 3.3: Bacterial community structures of the 45 sand samples. The percent abundance of each bacterial order in a sample is shown. Sequences were classified using Mothur and the Silva Gold 16S rRNA gene database with a confidence threshold of 80%. Sample codes include the month, time (before 0 and 1-168 h after wastewater was added) and sand fraction (C= unplanted control sand, PB= planted bulk sand, PR= rhizosphere sand). 94

Figure 3.4: Heat map of relative abundances of the most abundant (>1%) OTUs in each of the 45 samples and hierarchical cluster analysis presented as a double dendrogram. OTUs were classified to the family level where possible and clustered according to abundance, not phylogenetic similarity (x-axis). Sample code indicates sampling month, and time of sampling, i.e. before (0h), and after (1-168 h) settled sewage was added to the system. Sand fractions: C= unplanted control sand, PB=planted bulk sand, PR=rhizosphere sand..... 95

Figure 3.5: Non-random OTU co-occurrence networks and correlations with environmental variables. Spearman correlations with an r value >= 0.7 (solid edges) and <= -0.7 (dashed edges) at a confidence level of 95% were used to make the networks. Total carbon (C), carbon/ammonium ratio (C:NH_4), Chao richness, faecal coliforms (FC), nitrate (NO_3^-), phosphorus (P), Shannon diversity, and sulphur (S). FAME-chain length:number of double bonds; Me- methyl side chains. A=unplanted control sand, B= planted bulk sand, C=rhizosphere sand. Squares and circles indicate persistent (occurred in >80 % of samples), and transient OTUs (<80 %) respectively. 96

Figure 3.6: Comparison of FAME and OTU analysis of Gram negative, Gram positive and actinobacterial (which forms part of the Gram positive FAMEs) populations between the three sand fractions over a week. A=unplanted control sand, B= planted

bulk sand, C=rhizosphere sand. Lines are LOESS smoothed curves, and the shaded areas indicate a 95% confidence level. 97

Chapter 4

Figure 4.1: Potentially pathogenic yeast concentrations (A) and removal (B) after wastewater had passed through the planted and the unplanted side of the rhizofiltration system. Curves were smoothed with the LOESS non-parametric local regression statistic and shaded areas indicate a 95 % confidence interval..... 125

Figure 4.2: Variable importance plots for predictor variables from random forests regression used for predicting the concentration and relative abundance of potentially pathogenic yeasts (PPY). The increase in means square errors is the average increase in squared out of bag residuals when the variable is permuted. Higher values of increase in mean square errors indicate variables that are more important in the regression. 126

Figure 4.3: Relative abundance (%) of potentially pathogenic yeasts which occurred consistently in the wastewater across 20 Monthly sampling events from November 2011 to October 2013. A = Influent; B = Unplanted effluent; C = Planted effluent. 127

Figure 4.4: Non-metric multi dimensional scaling (NMDS) ordination plot of potentially pathogenic yeast community data (stress = 0.14), indicating significant ($p = 0.001$) differences in community composition in the formulated influent (TrI), planted side effluent (TrR) and unplanted side effluent (TrC). Circles = unplanted effluent, Squares = planted effluent, Triangles = formulated influent. Ellipses indicate standard error from point averages with a confidence limit of 0.95. Arrows of vectors points to the direction of most rapid change in the environmental variable, or the direction of the gradient, and the length of the arrow is proportional to the correlation between ordination and variable. Goodness-of-fit of vectors (r^2): Shannon's diversity index (H) = 0.381 ($p < 0.0001$); suspended solids (SS) = 0.241 ($p < 0.0001$); NH_4^+ = 0.129 ($p< 0.05$). Contours indicate the potentially pathogenic yeast concentration (log CFU.100 ml^{-1}). 128

Figure 4.5: Principal coordinate analysis of the generalized UniFrac alfa 0.5 distance matrix showing a significant difference (GPERMANOVA F-model = 11.08 ; $p = 0.044$) in fungal community composition between the planted and unplanted sides of the rhizofilter. Numbers indicate the sampling times: 0h before, 1h and 168h after wastewater was released onto the rhizofilter..... 129

Figure 4.6: Relative abundance of fungal OTUs in the total dataset and in each of the six samples. C= unplanted controls side, P=planted side of the rhizofilter. 130

Chapter 5

Figure 5.1: Conceptual model of treatment processes which may occur in the rhizofiltration system with information garnered in this study shown in green boxes. (a) Urban effluent contains numerous pollutants but the focus of this study was on

selected nutrients and microbial indicator organisms. Pollutant removal is hypothesized to be mediated by microbial biofilms (b) on surfaces such as the sand media (c) and plant roots (d) within the predominantly aerobic rhizofilter matrix. Biofilm activity is a function of biofilm thickness diversity and composition which is influenced by the redox potential (Eh), physical properties such as the sorption capacity (e) of the system and plant root exudates (d). The effect of oxygen release by the plant root may be negligible due the high aeration rate of the filter media. Organic carbon from the urban effluent (COD) and the ratio of recalcitrant to labile compounds (d) promotes and selects for a heterotrophic community with a high relative abundance of actinomycetes and α-Proteobacteria (f). Respiration by the heterotrophs produces CO_2 that is used as an inorganic carbon source by chemolithoautotrophs (g) which are responsible for nitrogen (nitrification by Nitrospiraceae) and sulphur cycles in the system (h). The products of the nutrient cycles could also influence biofilm characteristics. The biomass ratio between these two groups of bacteria is a function of their respective temperature dependant growth rates. Redox gradients in biofilms (c) and the rhizosphere (d) exist due to microbial respiration and differential spatial and temporal release of oxygen by the plant roots. This may allow for both aerobic and (restricted) anaerobic pollutant removal processes. All bacterial pathogens are heterotrophs (f) and thus, at some stage, form part of the heterotroph community in the rhizosphere. Enteric bacteria and potentially pathogenic yeasts had a low relative abundance and did not proliferate in the filter media. However potential pathogens within the genus *Mycobacterium* may occur in the rhizofilter. 147

Supplementary materials

Chapter 3

Figure S3.1: Relative abundance of phospholipid fatty acids. C=control, PB=planted bulk sand, PR=rhizosphere sand. 152

Figure S3.2: Boxplots of the relative abundance of the Actinomycetales before (0h) and after (1 -168 h) simulated urban effluent was added to the system and in the three sand fractions (C= unplanted control sand, PB=planted bulk sand, PR= rhizosphere sand. 153

Figure S3.3: Highly abundant species which occurred frequently in the respective sand fractions and their relative abundance (number of sequences). Species were identified by selecting representative sequences from the indicator OTUs (MOTHUR indicator species algorithm) and aligning them to sequences on the NCBI database using the BLAST tool. C=unplanted control; PB=planted bulk sand; PR=rhizosphere sand 154

Figure S3.4: Change in faecal coliform and *Salmonella/Shigella* concentrations in the planted rhizofilter bulk (RB), rhizosphere (RR), and unplanted control (C) sand fractions before (0 h) and after (1-168 h) the addition of simulated urban effluent. Values are the mean of triplicate samples taken over a period three months. Whiskers indicate standard error of the means. 155

Chapter 4

Figure S4.1: Colony morphology of potentially pathogenic yeast strains, representing different species, growing on molybdate agar. The photographs show the colonies from the top (A) and bottom (B) of the Petri dish. Strains: *Candida albicans* (CAB8908), *Candida glabrata* (CAB192), *Candida krusei* (CAB83), *Candida lusitaniae* (CAB76), *Candida tropicalis* (CAB632), *Candida utilis* (CAB78), *Pichia rhodanenis* (CAB77), and *Saccharomyces cerevisiae* (CAB618)..... 165

Figure S4.2: Sequence length distribution across the entire qaulity filtered sequence dataset. 166

List of tables

Chapter 2

Table 2.1: Summary of means of microbiological, physical and chemical data from weekly sampling events. 65

Table 2.2: Total quantity of pollutants adsorbed (qtotal) and removed determined by calculating the area under the breakthrough curves and comparing it to the total pollutant load (m_{total}) that percolated through the filter medium on the planted (experiment) and unplanted (control) sides of the rhizofilter..... 66

Chapter 4

Table 4.1: Means and standard deviations of potentially pathogenic yeast (PPY), physical and chemical data from twenty sampling events occurring monthly from November 2011 until October 2013. 131

Table 4.2: Significant Spearman rank order correlations (r-value) among potentially pathogenic yeasts (PPY) concentration, PPY removal (PPY-R) and the physico-chemical parameters. 132

Table 4.3: Dominant yeasts isolated during November 2011 to October 2013 from the formulated influent and effluent of both the unplanted (control) and planted (experiment) sides of the rhizofilter. 133

Table 4.4: Mean and standard deviation of the diversity, richness and evenness of the fungal communities in the planted and unplanted sides of the rhizofiltration system. No significant difference (Kruskal Wallis-ANOVA $p>0.05$) was observed between the planted (experiment) and unplanted (control) side in any of the indices. 134

Supplementary materials

Chapter 3

Table S3.1: Fusion primers used for ion torrent sequence analysis of the V4-V5 hypervariable regions of the 16S rRNA gene. 156

Table S3.2: Summary of sequence processing 157

Table S3.3: Microbial indicator and physico-chemical properties of influent and effluents of the unplanted and planted side of the rhizofiltration system. Values are the mean of triplicate samples and the standard deviation from the mean..... 158

Chapter 4

Table S4.1: Barcoded primers sequences used for multiplex high throughput metagenome sequencing of the fungal ITS1 region using the IonTorrent personal genome machine. 167

Table S4.2: Number of sequences before and after quality control steps. C = unplanted control sand, P = planted sand, and time of sampling before (0 h) and after the addition of simulated urban effluent (1 and 168 h). 168

Abbreviations

BMPs - Best Management Practices

BOD - Biological Oxygen Demand

COD - Chemical Oxygen Demand

Cl^- - Chloride

Eh - Redox Potential

FC - Faecal Coliforms

FAMEs - Fatty Acid Methyl Esters

HLR - Hydraulic Loading Rate

HRT - Hydraulic Retention Time

HTS - High Throughput Sequencing

H_2S - Hydrogen sulphide

$H_2S_nO_6$ - Polythionates

LOESS - Locally Estimated Scatterplot Smoothing

N_2 - Nitrogen gas

NH_4^+ - Ammonium

NMDS - Non-Metric Multidimensional Scaling

NO_2^- - Nitrite

NO_3^- - Nitrate

N_2O - Nitrous oxide

NOB - Nitrite Oxidizing Bacteria

NRB - Nitrate Reducing Bacteria

O_2 - Oxygen

OTUs - Operational Taxonomic Units

PCR – Polymerase Chain Reaction

PGM - Personal Genome Machine

PLFA - Phospholipid Fatty Acids

PO_4^{3-} - Phosphate

PPY - Potentially Pathogenic Yeasts

S^{2-} - Sulphide

SL - S*almonella/Shigella*

SO_3^{2-} - Sulphite

SO_4^{2-} - Sulphate

$S_2O_3^{2-}$ - Thiosulphate

SRB - Sulphate reducing bacteria

SS - Suspended Solids

WWTW - Waste Water Treatment Works

Equations

Chapter 2

Total effluent volume (eq. 1)

$$V_{eff} = Qt_{total}$$

Quantity adsorbed (eq. 2)

$$q_{total} = Q \int_{t=0}^{t=45\,min} C_{ad}\, dt$$

Total pollutant load (eq. 3)

$$m_{total} = C_o Q t_{total}$$

Percentage removal (eq. 4)

$$Removal = \frac{q_{total}}{m_{total}} x\,100$$

Chapter 1

Microbial processes in a rhizofilter treating polluted urban runoff: A review and conceptual model

1 Introduction and background

Worldwide, increasing urbanization and land development has led to pollution of surface waters through urban runoff (Fletcher et al., 2008; Göbel et al., 2007; Jamwal et al., 2008). Polluted urban runoff is a complicated mixture of numerous chemicals and organisms. It has been identified as a contributor to visible matter, suspended solids, oxygen demanding materials, pathogenic microorganisms, heavy metals, nutrients and toxic anthropogenic chemicals in surface waters (Behera et al., 2006; Göbel et al., 2007; Hathaway and Hunt, 2010; Jackson et al., 2009; Okafo et al., 2003). The composition of urban effluent is strongly affected by land use, seasons, climate and associated rainfall, vegetation cover, proportion of impermeable surfaces, traffic densities, population densities and other landscape features (Al Bakri et al., 2008; Dwight et al., 2011). A major source of non-point pollution contributing to urban effluent, especially in developing countries, are large populations residing in informal settlements which are often without any sanitation infrastructure (Jackson et al., 2009; Sverdlik, 2011). Furthermore, the rapid development of informal settlements on the outskirts of cities and towns has increased the amount of urban runoff that flows into various water systems (Jackson et al., 2009; Paulse et al. 2009). Inadequate infrastructure and facilities in these areas result in many residents using the riverbanks, or even the river itself, for ablutions. Moreover, ever increasing industrialization and illegal industrial discharges into river systems, further compromise water quality and threaten already fragile ecosystems (Bavor and Waters, 2007; Ramond et al., 2012).

A number of engineered systems, commonly referred to as best management practices (BMPs), were developed to remove pollutants from nonpoint sources (Beutel and Larson, 2014). These BMPs can be defined as the components of the drainage pathway between the source of runoff and a stormwater discharge location that affect the volume, timing,

and/or quality of runoff (Granato, 2014). Settling and filtration are usually the primary water quality treatment mechanisms that form the basis for reductions in influent concentrations for many constituents in commonly used BMP designs. However, chemical and biological processes are increasingly being incorporated into BMP designs to enhance treatment of runoff constituents (Granato, 2014). The application of various biofiltration technologies for the treatment of urban effluent has received increasing attention in the last decade (Beutel and Larson, 2014; Gregory et al., 2010; Zhu and Chen, 2001). However, it is still difficult to explain the behaviour of a biofilter. The design parameters required for the assembly of efficient biofilters have been relatively well formulated with respect to gross properties, including filter medium and flow rate (Brown et al., 2000; Iasur-Kruh et al., 2010). In contrast microorganisms occupying BMP systems are less explored, despite their major role in contaminant reduction (Iasur-Kruh et al., 2010). The full complexity of the interactions between microorganisms and the biotic as well as abiotic properties of BMP habitats are poorly understood and remain the focus of much research (Gregory et al., 2010).

Of particular interest in this review are the pollutant removal mechanisms and microbial community dynamics of constructed filtration systems that mimic natural riparian zones. Riparian zones are the interface between terrestrial and freshwater ecosystems. They are analogous to a semipermeable membrane regulating the flow of energy and material between adjacent environmental patches. These interfaces between terrestrial and aquatic environments are some of the most diverse, dynamic and complex biophysical habitats on the terrestrial portion of the earth (Naiman and Decamps, 1997). The ecosystem services they provide include the control of sediment and chemical transport into channel streams, nutrient sinks and hydrological buffers (Lowrance et al., 1984; O'Neil and Gordon, 1994). The biological processes occurring in these environments are influenced by the adjacent

ecological patches and resources which vary widely over spatial and temporal scales. Similarly, ecosystems within engineered riparian zones may be influenced by the stochastic nature of urban effluent events and composition. This makes it difficult to design systems which perform predictably and efficiently. Additionally, many of the current design criteria are based on empirically determined performance characteristics of constructed wetlands which may not be generally applicable (Rousseau et al., 2004)

Recent designs of constructed riparian zones included rhizofiltration as a pollutant treatment mechanism (Wilsenach et al., 2014). Rhizofiltration is a type of phytoremediation that uses plant roots and their associated microbial communities to remove pollutants from water through phytoextraction and biodegradation (Arthur et al., 2005; Veselý et al., 2011). Originally developed as an environmentally acceptable manner of removing heavy metals from aqueous matrices, rhizofiltration was recently evaluated as an option for the removal of microbial pathogens, nutrients and anthropogenic chemicals from urban runoff (Wilsenach et al., 2014). The rhizofilter design of Wilsenach and co-workers (2014) incorporated aspects of roughing filters, vegetated filter strips and constructed wetlands, to mimic the filtration effect of riparian zones (Figure 1.1).

Conceptual models, based on process mechanisms that are derived from experimental research, can be used in the development of design and operational strategies (Werker et al., 2002). Therefore the aim of this study was to review current knowledge of microbial pollutant treatment mechanisms in various types of biofiltration systems, e.g. constructed wetlands, trickling filters and rhizofilters. Information about these processes subsequently enabled us to form hypotheses about the relevant pollutant removal mechanisms that can be expected to occur in a rhizofiltration system.

2 Conceptual model development

The model proposed here attempts to represent the microbial communities and processes occurring in the pilot scale rhizofiltration system (Figure 1.1) designed by Wilsenach and co-workers (2014) as well as in similar BMPs. The conceptual model review starts with a description of the habitat that was created in a pilot scale rhizofilter. Secondly, hypotheses are formed on the microbial assemblages that may occur in rhizofilters. Finally, factors that may influence the microbial community composition and their related function are discussed.

The pollutant removal processes in engineered and natural environments are mediated by vegetation, soils and microbial communities that together act as a biofilter. During these processes, interdependant physical, chemical and biological dynamics contribute to nutrient and pathogen removal (Faulwetter et al., 2009). Physical factors include mechanical filtration, straining, adsorption and sedimentation (Brown et al., 2000). Chemical factors include redox potential, UV radiation, exposure to biocides secreted by some plants, as well as adsorption to organic matter. Biological removal factors include antibiosis, syntrophy, predation by nematodes, protozoa and zooplankton, attack by lytic bacteria, viruses, as well as natural die-off (Graves and Weaver, 2010).

The focus of this review will be on processes involved in the removal of pollutants like ammonia (NH_4^+), oxygen demanding substances represented by the chemical oxygen demand (COD), sulphate (SO_4^{2-}), phosphate (PO_4^{3-}) and indicator organisms including faecal coliforms, as well as *Salmonella/Shigella.* The ultimate goal is to integrate this information in a design framework to optimally construct rhizofiltration systems. While it is not possible to optimize conditions for all beneficial species, basic habitat requirements for all microbes include a substrate to colonize (e.g., soil, plant roots, or leaf surfaces),

appropriate nutrients (including carbon and nitrogen sources), absence of toxins, and sufficient moisture.

3 Biosystems engineering of riparian zones

At the beginning of the 20th century researchers discovered that the land water interface reduces nutrient movements to streams by capturing sediment and sediment-bound pollutants transported in agricultural and urban runoff (Karr & Schlosser 1978; Naiman & Decamps 1997). Since then BMP designs have been created to mimic this natural process. An example of such a system specifically aimed at mimicking riparian ecology is the rhizofilter design of Wilsenach and co-workers (2014). This system consisted of a coarse sand filter bed on top of two separate layers of gravel, and large stones (Figure 1.1). Additionally, commonly occurring helophytes (*Typha capensis* and *Phragmites australis*) were planted in the sand. This rhizofiltration system was based on vertical flow constructed wetlands, trickling and sand filters. The design allowed for vertical flow conditions through the planted sand medium, allowing atmospheric gases to permeate the sediment matrix, creating unsaturated oxygen rich conditions (Faulwetter et al., 2009; Ramond et al., 2012). Furthermore, the crushed rocks and large stones underneath the sand layer of the rhizofilter facilitated oxygen transfer throughout the entire sand layer.

An important consideration when selecting the filter media of biofilters is the adsorption capacity and bioregeneration capacity thereof. Adsorption to the surfaces within the filter bed is an important mechanism by which nutrients are removed (Sakadevan and Bavor, 1998). Additionally, microorganisms show preferential adhesion on different substrates (Donlan, 2002). Selection of a filter medium tailored to the wastewater composition and hydrological properties in the area, in which it will be applied, greatly improves the performance characteristics of BMPs.

4 Bacterial populations in BMPs

Microbial communities play a key role in the biogeochemical cycles of ecosystems and the presence of a stable microbial community is generally considered to be a critical factor for maintaining ecosystem stability, nutrient cycling efficiencies and long term sustainability (Ramond et al., 2012). Thus, it is crucial to understand the associations between the composition and diversity of microbial communities and the environmental parameters affecting these ecosystems. This knowledge can improve the design and management of constructed wetlands and thus enhance the efficiency of water purification (Truu et al., 2009). Assumptions about the microbial populations and their function within BMPs were originally based on microbial processes known to occur in other wastewater treatment systems and/or natural wetlands (Faulwetter et al., 2009). However, knowledge about these processes may not be generally applicable to all biofilter BMP designs and the large array of environmental conditions in which these technologies may be constructed.

Despite the contention that microbial communities in BMPs are important for the removal of pollutants from wastewater, few studies have analysed their composition. Using culturing techniques Huang and co-workers (2012) found that, in three horizontal subsurface flow wetlands, bacteria were the most numerous, followed by actinomycetes and fungi. Molecular approaches such as denaturing gradient gel electrophoresis (DGGE), and more recently next generation high throughput sequencing (HTS), allowed for higher resolution and better taxonomic identification of microbial communities. Studies on microcosms and full scale constructed wetlands, aimed at describing microbial communities using molecular methods, showed that the dominant bacterial phyla represented diverse bacterial lineages (Iasur-Kruh et al., 2010; Ligi et al., 2013; Peralta et al., 2013). Based on this research, bacterial phyla that may occur in constructed wetlands include the *Acidobacteria, Actinobacteria, Bacteriodetes, Chloroflexi, Firmicutes,* the α, β

and *δ* subdivisions of the *Proteobacteria* as well as the *Verrucomicrobia.* However, to obtain a better understanding of the functional capabilities of these bacteria, genus or family specific information is needed.

Previously, specific genetic evidence was found for the presence of nitrifying bacteria such as *Nitrosomonas, Nitrospira* and *Nitrobacter,* in constructed wetlands. Furthermore, constructed wetlands may contain bacteriovores and *Bdellovibrio*-like organisms (Iasur-Kruh et al., 2010). Predation by these bacteria and protozoa were found to play an important role in the removal of *Escherichia coli* from a vegetated sand column (Wand et al., 2007). However, it is challenging to quantify the link between the molecular characteristics of microbial diversity, activity and ecosystem function (Schmidt et al., 2011).

One of the hurdles to quantitatively relate microbial genomics to ecosystem function is a lack of understanding the functional redundancy of microbial populations. This hurdle can in part be overcome by using next generation high throughput sequencing which has the advantage in giving us in depth, high resolution data on the relative abundances of microbial taxa in an environment (Adams et al., 2013; Barberán et al., 2012; Di Bella et al., 2013; Ligi et al., 2013). When combined with measuring physico-chemical characteristics of the environment these bacteria occupy, we may be able to formulate hypotheses on how these parameters effect the microbial population and vice versa.

4.1 Distribution of bacteria in BMP filter media

When considering microbial communities it is important to realize that they may be heterogeneously distributed within a filter column. For example, in vertical flow wetlands direct microbial measurements showed that the first 5 to 10 cm of the filter media

contained the highest microbial density and activity (Ragusa et al., 2004; Tietz et al., 2007). This was probably caused by the high nutrient and oxygen supply in the upper zone of these wetlands (Tietz et al., 2007). However, using single strand conformation polymorphism, Vacca and co-workers (2005) found that microbial diversity in vertical filters, were distributed more or less homogenously over different depths. They also found that, in horizontal subsurface flow constructed wetlands, the microbial diversity was higher in the deeper parts. In addition, their results indicated that microbial community composition may be determined by the volumetric water content of the filter media which affects bacterial adsorption. Furthermore, it is known that bacterial transport by unsaturated flow is affected by adsorption to the air-water interfaces and to the solid phase (Powelson and Mills, 2001). The combination of flow regime, sorption processes, oxygen concentrations and related redox potentials are thus the primary factors that could affect microbial population diversity and composition in biofilters.

4.2 Biofilm processes

Surfaces within BMPs are covered with biofilms which are critical for nutrient conversion, especially in systems with a short hydraulic retention time (HRT), such as rhizofilters, in which planktonic cells could be washed out (Harremoës and Henze, 2002; Iasur-Kruh et al., 2010). Biofilm formation on solid surfaces has been investigated intensively and the processes involved are understood relatively well (Karatan and Watnick, 2009). Solid surfaces have a positive effect on bacterial activity, which may be due to fixed cultures being more resistant than planktonic cells with regard to changes in environmental conditions such as temperature, pH, nutrient concentrations, metabolic products and toxic substances (Lazarova and Manem, 1995). Planted BMPs offer mainly two types of surfaces, namely the filter medium particles and the plant roots.

Microbial biofilm formation and substrate degradation within wastewater is a complex process influenced by many factors including the wastewater characteristics (nature of substrate, nutrient ratio and environmental conditions), operational factors (inoculation, organic loading rate, hydrodynamics) and the support medium (Lessard and Bihan, 2003). In particular, the adsorption of organic polymers and proteins improves biofilm growth more strongly than nutrient adsorption. Moreover, biofilm composition is a function not only of physicochemical conditions, but also of microbial phenotypic characteristics. For example, the microbial consortia within biofilms may contain some bacterial strains that form microcolonies as homogenous layers, while others may develop vertical colonies on solid media (Lazarova and Manem, 1995).

Two important parameters for the successful operation and control of fixed film processes in wastewater treatment are biofilm composition and activity (Lazarova and Manem, 1995). Bacterial activity, and ultimately biofilter performance, is affected by biofilm thickness which affects rate limiting steps such as diffusive transport processes and biomass efficiency (Harremoës & Henze, 2002). Biofilm thickness in turn, is determined by the equilibrium between microbial growth and sloughing off through hydraulic erosion, processes which are respectively determined by the wastewater nutrient content and the flow characteristics of the system. Nutrient removal mechanisms within a biofilm are dependent on transport of oxygen and substrates from the water to the biofilm and within the biofilm, oxidation of the substrate, and diffusion of by-products back to the wastewater. A stable thin and active biofilm therefore offers numerous advantages in water and wastewater treatment (Lazarova and Manem, 1995). The development of technologies based on the concept of attached growth therefore necessitates in-depth knowledge, not only of the process behaviour, but also of microbiology and biofilm formation (Lessard and Bihan, 2003).

4.3 Effect of plants

Plants are a dominant feature in many BMP designs and it was shown by several authors that they improve pollutant removal (Faulwetter et al., 2009; Fraser et al., 2004; Picard et al., 2005). Both physical and chemical mechanisms may be involved in pollutant removal. For example the presence of plants in biofilters helps prevent clogging by their movement and growth (Brix and Arias, 2005). In soil it was found that plant roots could “prime” microbial activity with carbon rich root inputs leading to more rapid decomposition of older organic matter (Schmidt et al., 2011). Moreover, carbon released by plant roots, is retained in soils much more efficiently than carbon originating from other sources (Rasse et al., 2005; Schmidt et al., 2011). Similar to plant-soil interactions, plant mediated processes in BMPs may aid in the removal of recalcitrant carbon from wastewater. For example, Kurzbaum et al. (2010) evaluated the relative contribution of the different components of a low organic load constructed wetland system to remove phenol. They found that the component which contributed most to phenol removal was the plant roots and their associated biofilm bacteria. Therefore, carbon allocation via plant root systems may play an important role in carbon and microbial community dynamics within filter media contributing to pollutant removal. Also, the rhizosphere regions of subsurface constructed wetlands are known to harbour greater microbial density, activity and diversity than unplanted wetlands (Faulwetter et al., 2009). Therefore, microbes associated with plant roots are subjected to selective pressures created by the plant, which is determined by the plant species, genotype, and physiological state of the plant, as well as the presence of other microbial species (Danhorn and Fuqua, 2007).

5 Factors that influence biofilter microbial communities and performance

5.1 Nutrients in urban effluent

The main source of nutrients for microbial communities in engineered ecosystems is the wastewater they receive. Therefore, the chemical composition of wastewater may exert selective pressure on these microbial communities (Hibbing et al., 2010). Urban effluent often contains large quantities of organic material collectively measured and expressed as the COD (Jamwal et al., 2008; Göbel et al., 2007). Several potential organic substrates for microbial growth, which differ in their biodegradability, constitute the COD of wastewater. This includes aquatic humic substances, proteins, lipids, carbohydrates, fats and oils as well as polycyclic aromatic hydrocarbons.

Nitrogen and phosphorus are often the limiting nutrients in many ecosystems (De-Bashan & Bashan 2004). However, compounds containing these nutrients are concentrated in wastewaters due to discharge of household chemicals, fertilizers and organic material including faecal matter. Ammonium (NH_4^+), nitrate (NO_3^-), and orthophosphate (PO_4^{3-}) are readily available for uptake by algae and many other organisms causing blooms in aquatic ecosystems threatening the health of thereof (Taylor et al., 2005). Sulphate (SO_4^{2-}), a normal constituent of domestic wastewater, is one of the most universal anions occurring in rainfall, especially in air masses that have encountered metropolitan areas. Wastewater treatment works may also use SO_4^{2-} metal salts as a coagulant, which may be discharged with the final effluent into natural water channels (Alias & Assari 2007; Wiessner et al. 2005). The concentrations of SO_4^{2-} in wastewater can vary from only a few milligrams per litre to hundreds of milligrams per litre.

High concentrations of available organic carbon and growth limiting nutrients such as nitrogen and phosphorus in urban runoff (Taylor et al., 2005), could select for copiotrophic organisms in BMPs. These organisms are characterized by high growth and nutrient uptake rates compared to oligotrophs which have slow growth rates and high substrate affinities. However, similar to soil microbial communities a continuum of microorganisms differing in carbon requirements may exist in BMPs, ranging from oligotrophs to copiotrophs (Semenov, 1991). It must me noted however, that the recalcitrance of the organic carbon in urban effluent may be a major regulating factor in microbial community composition by affecting the amount energy available for growth and oxidation-reduction characteristics (Hu et al., 1999).

5.2 Redox potential

It is known that desirable microbial functional groups within BMPs can be selected for by manipulating the filter medium redox potential using design and/or operation variations (Faulwetter et al., 2009). Spatio-temporal variations of the redox potential (Eh) facilitates removal of pollutants by allowing functionally diverse microbial populations to be active through the successive stages often needed for pollutant degradation. Planted soil filters facilitate different redox states in the root zone which allow for simultaneously occurring microbial oxidation and reduction processes (Brune et al., 2000; Calheiros et al., 2007; Corbella et al., 2014; Wiessner et al., 2005).

Oxygen released by helophytes into the rhizosphere creates spatial and temporal micro-scale gradients of oxygen concentrations and redox states close to root surfaces (Wiessner et al., 2005). This redox gradient can range from ca. 500 mV very near the root surface, to ca. -250 mV at a distance of 1-20 mm from the root surface (Corbella et al., 2014; Wiessner et al., 2005). Furthermore the rhizosphere exhibits a mosaic of strong

redox gradients due to variations in oxygen loss according to root type and locations. The active growth regions of roots, particularly at the base of fine lateral rootlets, releases the most oxygen, while very little is lost from old roots and rhizomes (Armstrong and Armstong, 1990). This results in many ecological niches that promote a multitude of microbial processes. Many factors can affect the release of oxygen from roots including the macrophyte plant species, environmental conditions such as light intensity, humidity, temperature and the redox state of the filter medium (Armstrong and Armstong, 1990; Faulwetter et al., 2009; Wiessner et al., 2005)

Oxygen released by plant roots increases the respiration rate potential, as well as the microbial diversity in the rhizosphere (Gagnon et al., 2007). However, the influence of plant oxygen release in highly oxygenated systems may be negligible, due to high amounts of oxygen already permeating during the draining period (Faulwetter et al., 2009). Vertical flow wetlands and possibly rhizofiltration systems provide high levels of oxygen transfer favouring oxidation of organic carbon and reduced nitrogen species (Kadlec and Wallace, 2009). In contrast, the removal of certain pollutants through anaerobic processes such as denitrification and sulphate reduction may be inhibited in such systems (Faulwetter et al., 2009).

5.3 Mode of operation

The redox conditions of BMPs can also be managed by altering the hydraulic loading rate (HLR) and hydraulic retention time (HRT) (Faulwetter et al., 2009). The challenge is however to design a system that can adapt to stochastic stormwater events which may cause various inflow modes ranging from intermittent to continues flow (Kadlec and Wallace, 2009). Under differing inflow modes the system will behave differently. For example, laboratory studies revealed that constructed wetlands receiving wastewater

intermittently may show increased nitrogen and COD removal compared to conventional continues flow wetlands (Caselles-Osorio and García, 2007; Stein et al., 2003). These phenomena may perhaps be ascribed to changing redox conditions within these systems.

It is known that addition of wastewater to a biofilter results in decreased redox potential, which gradually recovers when the pollutants and water are removed (Faulwetter et al., 2009). Intermittent additions of wastewater in relatively rapid succession may therefore alter the environment within biofilters between reduced and oxidized conditions. This in turn may select for robust aerobic and facultative anaerobic microbial communities (Song et al., 2006; Zhao et al., 2010) which may contribute to mineralization within the system. In addition to inflow mode, the HRT and HLR greatly affect pollutant removal as these hydraulic parameters control the time of contact between microbial populations and the pollutants within a wetland system (Faulwetter et al., 2009; Toet et al., 2005). A longer HRT generates higher redox potentials and greater pollution removal (Allen et al., 2002; Dong et al., 2011; Headley et al., 2005). However, increasing the HRT of constructed wetlands often requires a larger surface area which is not always economically or geographically feasible (Dong et al., 2011).

5.4 Influence of seasons

It is generally known that microbial activity is linked to temperature, with bacterial growth and metabolic rates strongly reduced with decreasing temperature (Faulwetter et al., 2009). For example, early studies reported that nitrification may be inhibited below 10 °C while denitrification only occurs above 5 °C (Brodrick et al., 1988; Ilies and Mavinic, 2001; Werker et al., 2002). Additionally, plant oxygen transport by convective flow, which is assumed to be the most efficient mechanism within the plant, greatly decreases at low temperature (Armstrong and Armstong, 1990). For this reason, most early constructed

wetland design publications assumed poor pollution removal would occur during the winter season (Faulwetter et al., 2009). However, numerous studies, as reviewed by Kadlec and Reddy (2001), showed that seasonal temperature variation did not always affect COD and BOD removal. Several hypotheses were proposed to explain this better than expected winter removal efficiency, including the enhancement of aerobic (and more efficient) microbial degradation of organic matter due to increased redox potential with colder temperature (Allen et al., 2002; Stein & Hook, 2005).

6 Nutrient cycling

6.1 Organic carbon

Organic matter decomposition and nutrient cycling by microbial communities, which account for less than 5 % of the biomass in soil organic matter, are major processes in aquatic sediments (Cardinali et al., 2014; Nogaro et al., 2007). One of the most important factors impacting on organic matter mineralization in municipal wastewater treatment works is the input of degradable organic matter (Nogaro et al., 2007). For example, it was demonstrated that the input of particulate organic matter with a low carbon to nitrogen (C/N) ratio increased the metabolic and biogeochemical processes in hyporheic sediments (Crenshaw et al., 2002). Also, while studying the performance of a laboratory scale activated sludge reactor fed with synthetic wastewater influent, Han and co-workers (2010) found that increased COD loading rates at identical HRTs increased the microbial diversity of the system. Inversely, shorter HRTs supported fewer microbial species, resulting in the metabolism of fewer carbon sources (Han et al., 2010). The microbial processes in activated sludge systems are however very different from those in vegetated biofilters. Similar studies have not been done on biofilters, but the presence of plants that continuously leach organic carbon into the filter media may support a more consistent microbial community than the community with a filter devoid of plants.

Organic matter in water flowing through a biofilter may impact on the cycling of nutrients other than carbon, e.g. it was found that the nitrification rate of a biofilter is normally inhibited by the presence of organic matter (Zhu and Chen, 2001). Easily biodegradable organic matter in wastewater supports growth of heterotrophic bacteria, which compete with the autotrophic nitrifiers for oxygen, nutrients and space, especially in fixed film processes. Heterotrophic bacteria typically have a maximum growth rate of five times, and yields of two to three times more than that of nitrifiers (Zhu and Chen, 2001). Consequently, in a multi-species biofiltration process where both types of bacteria coexist, the heterotrophs are capable of utilizing more oxygen and space than the nitrifiers, and thus significantly inhibit nitrification when the oxygen supply and space are limited in a fixed film biofilter (Zhu and Chen, 2001).

Furthermore it was shown that, in multi-species biofilms, the percentage ammonia oxidizers and nitrite-oxidizers decreased with an increasing substrate C/N ratio, and the heterotrophs became more dominant as the C/N ratio increased (Ohashi et al., 1995). Satoh et al. (2000) found that an increase in the substrate C/N ratio (using acetate) immediately induced the interspecies competition for oxygen between ammonium oxidizing bacteria (AOB) and heterotrophs at the outer region of a biofilm (Satoh et al., 2000). As a result, the AOB population was reduced and ammonia oxidation was restrained. However, in the absence of an external carbon source, organic carbon produced by nitrifiers from inorganic carbon may be released in the filter medium. Therefore, these microbial groups also interact through the exchange of organic carbon (Kindaichi et al., 2004).

6.2 Nitrogen

For more than a century treatment systems containing aerobic biofilms were used to remove nitrogen from wastewater (Kindaichi et al., 2004; Wilsenach et al., 2014). The primary pathway for removal of nitrogen is considered to be nitrification, followed by the anaerobic process of denitrification. Nitrification is traditionally considered to be carried out predominantly by aerobic chemolithoautotrophic bacteria capable of using inorganic nitrogen as an energy source (Gregory et al., 2010). These bacteria are responsible for two successive aerobic reactions: firstly NH_4^+ is oxidized to NO_2^-, by AOB typified by the genus *Nitrosomonas,* and secondly the conversion of NO_2^- to NO_3^- by NO_2^- oxidizing bacteria (NOB) typified by the genus *Nitrobacter* (Prosser, 1989). Oxidation of NH_4^+ or NO_2^- provides the only source of energy for these organisms, and organic carbon is obtained by the fixation of carbon dioxide. Nitrifying bacteria, and the process of nitrification, are aerobic with optimal activity at mesophilic temperatures and neutral to alkaline conditions, with no growth or activity within an acidic environment. These organisms have low specific growth rates as well as growth yields due to relatively little energy gained from the oxidation of NH_4^+ to NO_3^- (Prosser, 1989, Satoh et al., 2000).

Ammonia oxidizing bacteria and NOB were previously delimited solely on physiological properties, i.e. the utilization of ammonia or nitrite oxidation as an energy source, and fixation of carbon dioxide for organic carbon (Prosser, 1989). Today, comparative 16 S rRNA gene sequence analyses of AOB species showed that *Nitrosococcus halophilus* and *Nitrosococcus oceani* belong to the Gammaproteobacteria, while the others form a monophyletic lineage within the Betaproteobacteria (Purkhold, 2003). Betaproteobacterial AOB encompasses the genera *Nitrosomonas* and *Nitrospira*. All members are Gram negative, but exhibit a wide range of morphologies, with regard to cell shape and arrangement of intracytoplasmic membranes. *Nitrosolobus* species are the most common

ammonia oxidizers in soil with a neutral pH, where as *Nitrospira* species dominate in acidic soils, and *Nitrosococcus* species are important in marine environments (Prosser, 1989).

Factors that can affect nitrification-denitrification in BMPs include the adsorption-desorption kinetics of the nitrogen compounds to the filter media and the flooding regime (Gregory et al., 2010). The rhizofilter was operated in a batch feeding mode (Figure 1.1; Wilsenach et al., 2014). This results in alterations between aerobic and anaerobic conditions during the drainage and flooding periods respectively. During flooding NH_4^+ in the wastewater binds to cation exchange sites (Toet et al., 2005). In the subsequent drained periods nitrifying bacteria oxidize the NH_4^+ provided that oxygen and inorganic carbon is present. When the BMP is flooded again the NO_3^- may be reduced by denitrifiers to gaseous nitrogen products if the hydraulic retention time is sufficiently long for anaerobic conditions to persist. During this process cation exchange sites become available again and the products are release into the water. Clearly, in this scenario, the amount of NH_4^+ that can be removed is limited by the adsorption capacity of the filter medium.

The concentration of NH_4^+ in urban effluent also plays a regulatory role in the nitrification process (Gregory et al., 2010). Shifts in the nitrifying communities within a biofilter can occur with changes in NH_4^+ concentrations. Also, high NH_4^+ concentrations were observed to cause accumulation of NO_2^- in biofilters. Elevated NO_2^- levels are known to inhibit both AOB and NOB (Gregory et al., 2010). Although the removal of NH_4^+ is often the main focus of treatment systems other forms of inorganic (NO_3^-, NO_2^-) and organic (dissolved, particulate) nitrogen may occur in urban effluent (Taylor et al., 2005). The composition of nitrogen containing compounds prior to entering treatment systems, such as BMPs, is an important consideration for enhancing current treatment capabilities. Furthermore, aquatic

macrophytes were shown to improve nitrogen removal via coupled nitrification-denitrification processes (Risgaard-Peterson and Jensen, 1997). Plants may also assimilate the NO_3^- formed by nitrification (Arthure et al, 2005; Fraser et al., 2004; Jin et al., 2002). Similarly, heterotrophs may remove NH_4^+ and NO_3^- through assimilatory and dissimilatory reduction processes (Brown et al., 2005).

6.3 Phosphate

Removal of phosphorus, especially PO_4^{3-}, from urban effluent is important to protect lakes and other natural waters from eutrophication (Fuhs and Chen, 1975). BMPs are usually not designed to remove nutrients such as PO_4^{3-}, however; they do so indirectly because the ions are nutrients for plants (De-Bashan and Bashan, 2004). Phosphate removal in constructed wetlands can range from 60 to over 90 %. Gravel alone may provide significant improvement of effluent quality in wastewater treatment systems, but adding vegetation, especially the macrophyte *Typha* spp., further improves treatment efficiencies (De-Bashan and Bashan, 2004). Phosphate removal in a rhizofiltration system was found to have very low mean removal efficiency (< 2 %) (Wilsenach et al., 2014). This indicated that the filter media was mostly saturated with PO_4^{3-} ions and that the uptake/solubilization mechanisms were not quick enough to free space for PO_4^{3-} attachment before wastewater was added to the system. Performance of such a rhizofiltration system in removing PO_4^{3-} can possibly be enhanced by increasing the filter medium volume or by using a filter medium with a higher PO_4^{3-} sorption capacity. Also, frequent removal of plant leaves will remove the phosphorus captured in the plant biomass and prevent PO_4^{3-} from re-entering the system due to leaching from decaying plant material.

6.4 Sulphate

Sulphur occurs in surface waters mainly in two forms: as SO_4^{2-} in aerobic waters, and as hydrogen sulfide (H_2S) in anaerobic waters. Within wetlands, sulphur may occur in a range of intermediate oxidation states e.g. SO_4^{2-}, sulfite (SO_3^{2-}), thiosulphate ($S_2O_3^{2-}$), polythionates ($H_2S_nO_6$) and sulphide (S^{2-}) (Truu et al., 2009; Wiessner et al., 2005). It was found that the variability of the redox state in the root zone may affect the fate of SO_4^{2-} and ultimately lead to the formation of S^{2-} (Wiessner et al., 2005). However, knowledge regarding the dynamics of the different oxidation states of sulphur and their interactions under gradient redox conditions in planted soil filters, is highly limited (Wiessner et al., 2005). Nevertheless, bacterial SO_4^{2-} reduction (BSR) to S^{2-} is widely considered to be the dominant process involved in SO_4^{2-} removal from constructed wetlands (Vymazal and Kröpfelová, 2009). Many environmental factors are known to influence BSR in constructed wetlands, including redox potential, dissolved oxygen, temperature, pH value, and type of organic carbon (Chen et al., 2014). Moreover, the sulphur cycle is closely linked to other element cycles, such as the carbon and nitrogen cycles (Muyzer and Stams, 2008). For example, Wiessner and co-workers (2005) found that SO_4^{2-} reduction reduced NH_4^+ and BOD removal in a laboratory scale continuously fed constructed wetland. They also showed that higher carbon loads increased the SO_4^{2-} reducing performance of the system.

Since rhizofiltration is predominantly aerobic, the anaerobic reduction of SO_4^{2-} is unlikely to occur at a sufficiently high rate to remove SO_4^{2-} from the bulk water during loading of the system. However, after wastewater percolated through the system, SO_4^{2-} reducing bacteria occurring at the anaerobic zone in the interface between the biofilm and the filter media surface, may be able to reduce the SO_4^{2-} that adsorbed to the biofilm surface. Sulphur, an essential element for life, is taken up as SO_4^{2-} and reduced to S^{2-}, which is then incorporated into sulphur containing amino acids and enzymes by microorganisms,

plants, and subsequently by animals (Muyzer and Stams, 2008). Decomposition of dead organisms in the absence of oxygen releases the sulphur again as H_2S. Sulphate reduction during drained periods of a rhizofilter may be an important mechanism by which a rhizofilter's capacity for SO_4^{2-} removal is regenerated, making space available on the filter medium for ion exchange when the next batch of wastewater is received. Previously, SO_4^{2-} removal by a rhizofilter was found to be relatively constant at concentrations ranging from 10-50 $mg.l^{-1}$ (Wilsenach et al., 2014).

7 Treatment of bacterial pathogens

It is not always economically and technically feasible to monitor pathogen levels in wastewater directly (Tamplin, 2003). As a result, it has become routine practice to monitor the levels of selected indicator organisms that are easy to detect and of which the numbers correlate with that of pathogens. The coliform group has been used worldwide as an indicator of faecal contamination (Toze, 1999). Faeces contain a variety of microorganisms that are present in concentrations as high as 10^{10} per gram. By far the most dominant intestinal organisms are anaerobic bacteria. Most of these species are not pathogenic to humans, but are essential for maintaining the normal functions of the gastrointestinal system (Tamplin, 2003).

Facultative anaerobic bacteria, also occurring in the gastrointestinal system, are mostly non-pathogenic; however, this group contains the majority of pathogens that cause human water-borne diseases, such as pathogenic strains of *Campylobacter, Salmonella, Shigella* and *Escherichia coli* (Hsu et al., 2011; Jenkins et al., 2008; Tamplin, 2003). The latter species forms part of the so-called total coliforms, which include aerobic and facultative anaerobic, Gram negative, non-spore-forming, rod shaped bacteria that ferment lactose with acid and gas production within 48 hours at 35 °C. In addition to *E. coli,* total coliforms

include members of the genera *Citrobacter, Enterobacter,* and *Klebsiella.* These coliforms are discharged in high numbers (2 x10^9 coliforms.day^{-1}.capita^{-1}) in human and animal faeces. However, not all strains representing these genera are of faecal origin (Tamplin, 2003). Not even the so-called faecal coliforms, including *E. coli* and *Klebsiella pneumonia,* which are known to be thermotolerant and able to ferment lactose at 44.5 °C in 24 hours, are specific to human or animal faeces. It is known that representatives of these species can be found not only in faeces, but also in different plant materials. Reports exist on the occurrence and growth of *K. pneumonia*, as well as other members of the *Enterobacteriaceae*, in natural vegetation including wetlands and woodlands (Berg et al., 2005; Tamplin, 2003;). In addition, Vacca and co-workers (2005) found that some representatives of the *Enterobacteriaceae* may form part of rhizosphere communities in constructed wetlands, and that the exudates of *Phragmites australis* or other competing bacteria in its rhizosphere do not cause a significant elimination of these enteric bacteria (Vacca et al., 2005). Today, many scientists and policymakers recognize the ubiquitous nature of coliform bacteria, and there have been important developments in the proposed utilization of more specific tests for measuring human faecal impact on water. These tests often include the use of the polymerase chain reaction (PCR) to amplify marker nucleotide sequences of pathogenic bacteria (e.g. *E. coli* O157:H7, *Salmonella, Campylobacter*), protozoa (e.g. *Cryptosporidium, Giardia*) and viruses (e.g. adenovirus, enterovirus) of enteric origin (Gironás et al., 2010; Toze, 1999).

8 Conclusion

Rhizofiltration can be a viable addition to current BMPs for the treatment of polluted urban effluent. Different variables were identified which could influence the performance of the rhizofilter, especially in terms of nutrient removal. These variables were included in a conceptual model (Figure 1.2) which could be used as a basis for future studies of

pollutant removal mechanisms in vegetated biofilters. Previous studies performed on numerous types of vegetated biofilters revealed that the microbial communities are strong determinants of performance. Research on the microbial communities occurring in the rhizosphere and their adaptive processes to stochastic pollutant loads may provide more insight into how to manipulate these processes through operational and design alterations. Moreover, microorganisms could be responsible for much of the bioregeneration of the filter medium allowing for consistent pollutant sorption to occur throughout the systems operational life.

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Figures

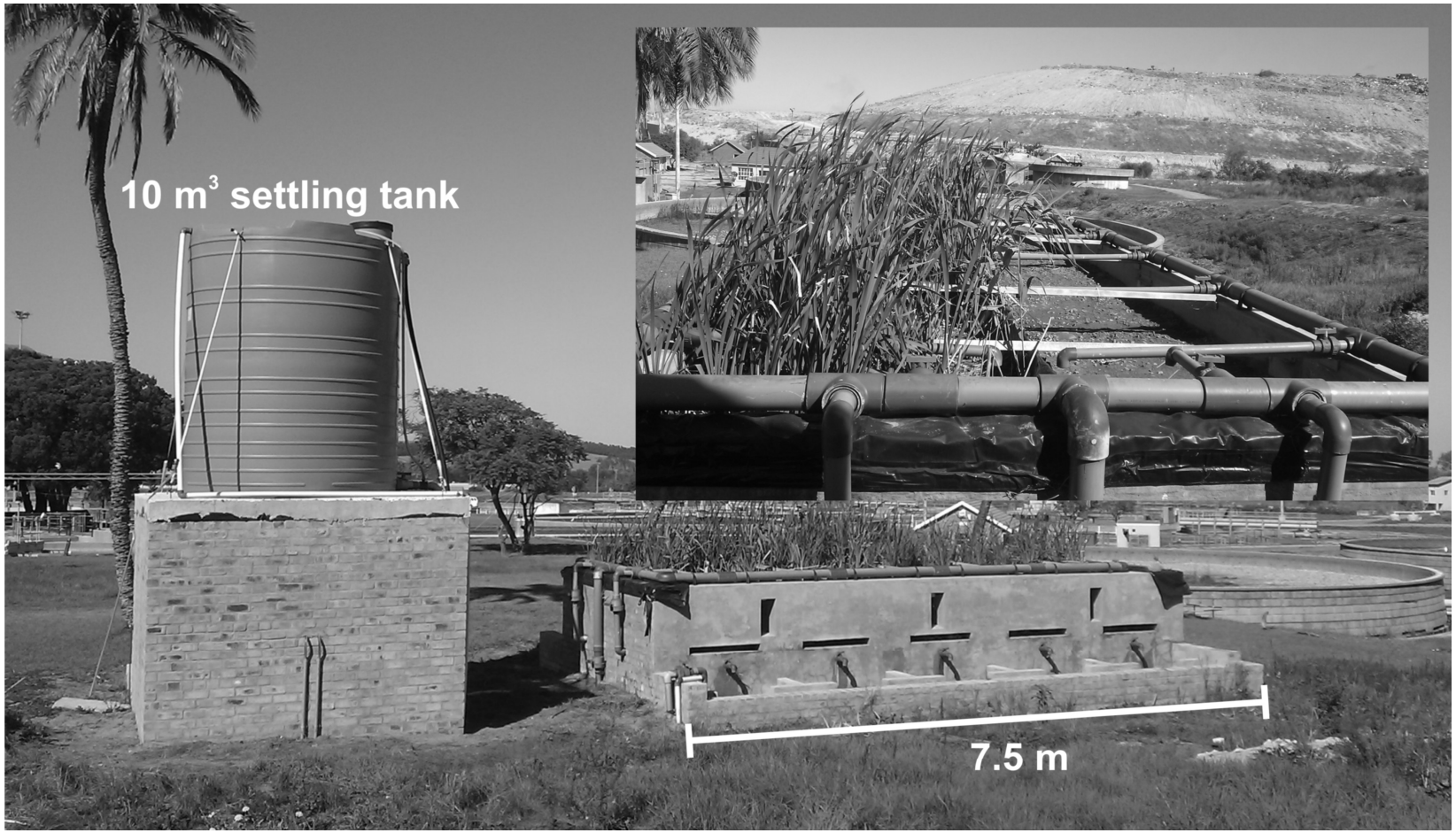

Figure 1.1: The rhizofilter experimental setup (Wilsenach et al., 2014). Settled municipal wastewater was pumped from a trickling filter distribution sump into the 10 000 L tank where it settled for a further 24 hours. Excessive solids were removed by flushing 2 000 L of the water prior to releasing the rest of the formulated influent (8 000 L) onto the planted and unplanted sides (insert) of the system at a rate of 7 $L.s^{-1}$. The water percolated vertically through the filter media for 45 minutes.

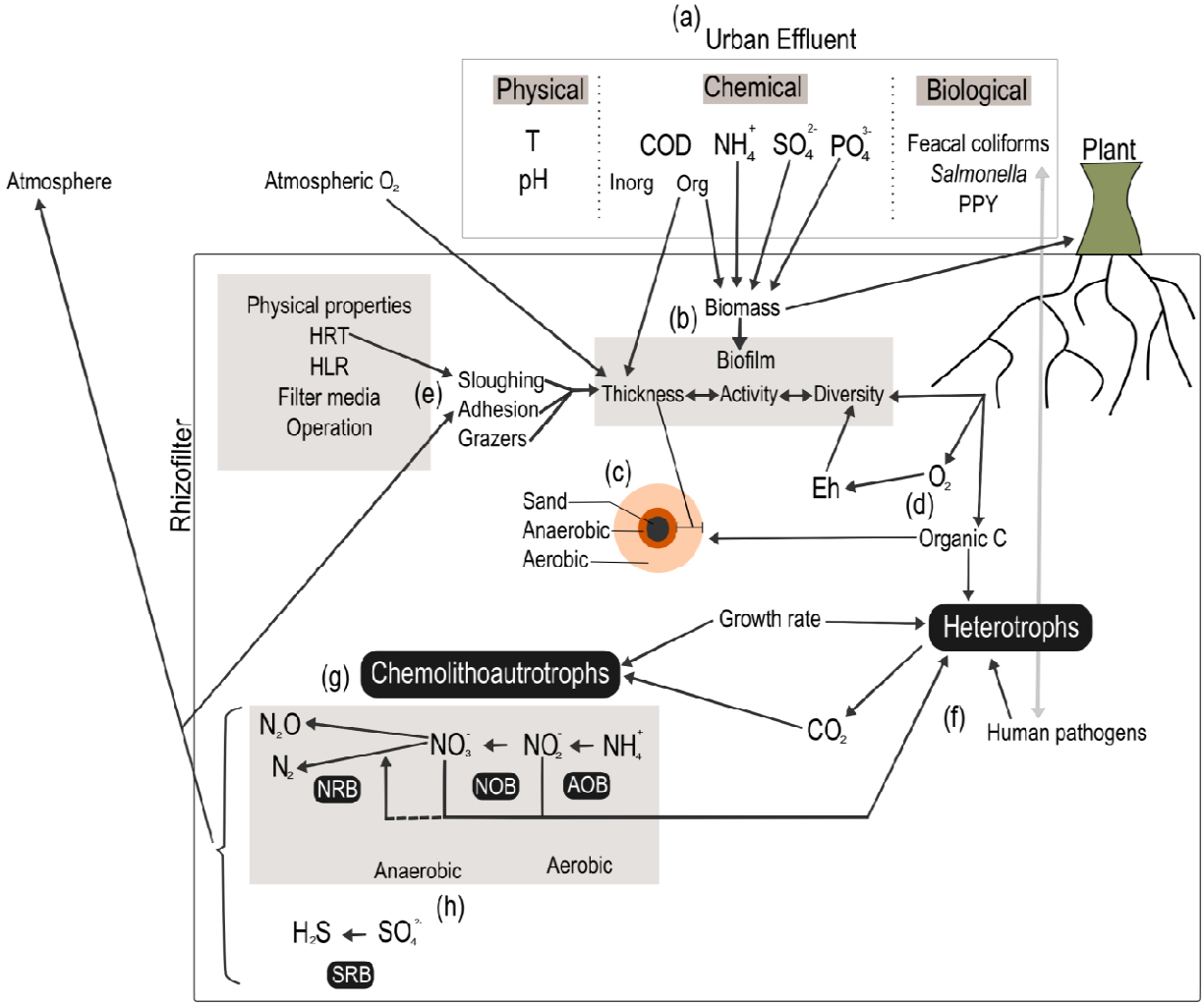

Figure 1.2: Conceptual model of treatment processes which may occur in the rhizofiltration system. (a) Urban effluent contains numerous pollutants but the focus of this study is on selected nutrients and microbial indicator organisms. Pollutant removal is hypothesized to be mediated by microbial biofilms (b) on surfaces such as the sand media (c) and plant roots (d) within the predominantly aerobic rhizofilter matrix. Biofilm activity is a function of biofilm thickness and diversity which is influenced by the redox potential (Eh), physical properties (e) of the system and plant root exudates including oxygen (d). Organic carbon from the urban effluent (COD) and released by the plant roots (d) promotes the growth of heterotrophs (f) that may be aerobic and/or facultative anaerobic. Respiration by the heterotrophs produces CO_2 that is used as an inorganic carbon source by chemolithoautotrophs (g) which are responsible for nitrogen and sulphur cycles in the system (h). The products of the nutrient cycles could also influence biofilm characteristics. The biomass ratio between these two groups of bacteria is a function of their respective temperature dependant growth rates. Redox gradients in biofilms (c) and the rhizosphere (d) exist due to microbial respiration and differential spatial and temporal release of oxygen by the plant roots. This may allow for both aerobic and (restricted) anaerobic pollutant removal processes. All bacterial pathogens are heterotrophs (f) and thus, at some stage, form part of the heterotroph community in the rhizosphere. Studying the dynamics within the heterotrophic community could lead to important insights in pathogen removal mechanisms.

Abbreviations: T = temperature; COD = chemical oxygen demand; Org = organic; HRT = hydraulic retention time; HLR = hydraulic loading rate, C = carbon; NRB = nitrate reducing bacteria; NOB = nitrite oxidizing bacteria; AOB = ammonia oxydizing bacteria; SRB = sulphate reducing bacteria; PPY = potentially pathogenic yeasts.

Chapter 2

The regeneration and sorption capacity of a pilot scale rhizofilter

1 Introduction

Nutrient and pathogen removal from wastewater by a rhizofilter is largely determined by the sorption capacity of the filter media (Cucarella and Renman, 2009). This entails both the adsorption of nutrients and organic materials to the mineral and biofilm surfaces, as well as the absorption of these pollutants by the biofilms (McLean et al., 2006). The liquid-solid phase adsorption, at continuous flow conditions, is recognized as a non-linear and multivariable process that can be analyzed with a breakthrough curve (Aksu et al., 2002; Aksu and Gönen, 2004; McGinley et al., 1996; Rojas-Mayorga et al., 2015; Slaney and Bhamidimarri, 1998). Such graphs describe the adsorption column dynamics and provide relevant information concerning key parameters that may influence the design, operation, as well as optimization of the separation system (Rojas-Mayorga et al., 2015). Breakthrough curves of pollutant sorption often exhibit a characteristic asymmetric sigmoidal profile, with varying degrees of steepness. The sorption capacity of a filtration system coupled with the regeneration of the filter medium ensures continual efficient removal of pollutants (Pagans, 2004). An important advantage of biofilters is that biological regeneration of the sorbent material can occur, thereby maintaining the sorption capacity of the filter media (Akar et al., 2013, Pagans, 2004). Aktas and co-workers (2007), defines bioregeneration, as the renewal of the sorption capacity of sorbents by microbial degradation, which leads to further sorption.

The bioregeneration capacity of a rhizofiltration system with regard to nutrient and pathogen removal has never been studied. Therefore, the first aim of this study was to evaluate the bioregeneration capacity of a rhizofilter system with regard to chemical and microbiological pollutants expected to occur in urban runoff. Secondly, we aimed to determine the sorption capacity of the system. Determining these facets of the rhizofilter,

could be valuable in optimizing the system thereby ensuring predictable and efficient removal of pollutants from urban runoff before it reaches river systems.

2 Materials and methods

2.1 Experimental setup

The experimental rhizofiltration system at Stellenbosch wastewater treatment works was employed for this study (Figure 1.1) Western Cape, South Africa (33,94335S-18,82425E). The region has a Mediterranean climate characterized by hot, dry summers and cool, moist winters (Di Castri, 1991). The LID-BMP (henceforth referred to as the rhizofilter) is an aboveground concrete structure 7.5 m long, with a depth of 1 m and width of 3 m. To test the hypothesis that plants select for specific microbial populations, the rhizofilter was divided lengthwise using a wire mesh covered by high density polyethylene sheets, to create two separate basins. Both basins were filled with a filter medium, which consisted of three ca. 3 m^3 layers of rocks and sand, ranging from coarse rocks (100 -120 mm diameter) at the bottom, to crushed rocks (19 - 25 mm diameter) in the middle, and a top layer of coarse river sand. One of the basins was evenly planted (the experimental side) with two helophyte species, namely *Typha capensis* (Rohrb.) and *Phragmites australis* (Cav.; Steud.), while the control side remained unplanted.

2.2 Biological regeneration / performance tests

The biological regeneration time was examined by conducting a weekly performance test for 10 weeks, after a pulse discharge of formulated influent onto the rhizofilter. Settled municipal wastewater was pumped into a 10 000 L tank and allowed to further settle overnight. Subsequently, the first (bottom) 2000 L was flushed to remove excessive solids and the remaining 8000 L of the formulated influent, was released onto the system through

two pipes each on the planted and unplanted sides (Figure 1.1).The formulated influent was allowed to percolate vertically through the system for a total duration of 45 minutes.

Effluent water was collected from two outlets on both the planted and unplanted sides of the rhizofilter in 500 ml sterilized glass bottles. Samples were also collected from the formulated influent. Nitrate (NO_3^-), ammonium (NH_4^+) and sulphate (SO_4^{2-}) concentrations in the influent and effluent samples were determined using a SEAL AutoAnalyser 3 HR (SEAL Analytical). The phosphate (PO_4^{3-}) concentrations were determined with inductively coupled plasma mass spectrometry (ICP-MS) analysis. The chemical oxygen demand (COD) was determined with a photometric kit (Spectroquant ®; Merck-Millipore) according to the manufacturer's instructions. The suspended solids concentration was determined by filtering the water sample through a standard glass-fibre filter (Bukhari, 2008). Subsequently, the residue retained on the filter was dried to a constant weight at 103-105 °C. The increase in weight of the filter was an indication of the total amount of suspended solids.

The faecal coliform and *Salmonella/Shigella* concentrations in the samples were determined using dilution (physiological saline solution) plates prepared with MacConkey (BioLab, Merck, Germany) and *Salmonella/Shigella* (Difco, Becton Dickinson and Company, NJ, USA) selective media respectively. Enumeration of the presumptive faecal coliforms was conducted after an incubation period of 24 h at 44.5 °C, while the numbers of presumptive *Salmonella* and *Shigella* on the plates were determined after an incubation period of 24 h at 37 °C. The coliphage concentrations in the samples were determined using the direct plaque assay method described by Baker et al. (2003). Enumeration of the coliphages was conducted after an incubation period of 18 h at 37 °C.

2.3 Sorption capacity

To determine the sorption capacity, the formulated influent was allowed to percolate vertically through the system for a total duration of 45 minutes, with influent and effluent samples taken in triplicate at 3 minute intervals. The sorption capacity of the rhizofiltration system was determined for the following chemical and biological pollutants: NH_4^+, COD, NO_3^-, PO_4^{3-}, SO_4^{2-}, suspended solids, faecal coliforms, *Salmonella/Shigella* and coliphages. Influent samples were collected from one of the overhead pipes and effluent samples were collected from two outlets on both the planted and unplanted sides in sterile 250 ml glass bottles.

Colorimetric tests were performed on the samples to determine the concentrations of the chemical pollutants NH_4^+ (Harwood and Huyser 1970), SO_4^{2-} (Brunt, 1985), NO_3^- (Doane and Horwáth, 2011) and PO_4^{3-} (John, 1970). The concentrations of COD, suspended solids, faecal coliforms, *Salmonella/Shigella* and coliphages were determined as described above.

2.4 Sorption capacity calculations

Breakthrough curves were used to indicate the loading and removal behavior of pollutants. The normalized concentration, defined by the ratio of the effluent pollutant concentrations (C) to the influent pollutant concentrations (C_o) was plotted as a function of the cumulative effluent volume (L) (Aksu et al., 2002; Aksu and Gönen, 2004).

The total effluent volume (V_{eff}) was calculated from Eq. (1):

$$V_{eff} = Qt_{total} \quad (1)$$

where t_{total} and Q are the total flow time (min) and percolation rate (L/min). The respective mean flow rates of the planted and unplanted sides were previously determined to be 96 and 84 L/min (Wilsenach et al., 2014). The V_{eff} values for the respective sides after 45 min were calculated to be 4 320 and 3 780 L. To determine the total quantity of adsorbed pollutants the area under the breakthrough curve (A) was calculated by integrating the adsorbed concentration (C_{ad}= C_i-C_e) versus t (min) plot from Eq (2):

$$q_{total} = Q \int_{t=0}^{t=45\,min} C_{ad}\,dt \quad (2)$$

The total pollutant load released onto the filter (m_{total}) was calculated from Eq. (3):

$$m_{total} = C_o Q t_{total} \quad (3)$$

Furthermore, the percentage pollutant removal with respect to flow volume was calculated from the ratio of adsorbed (q_{total}) to total load (m_{total}) of the pollutants Eq. (4):

$$Removal = \frac{q_{total}}{m_{total}} x\,100 \quad (4)$$

The locally estimated scatterplot smoothing (LOESS) breakthrough curves of the C/C_o value over the cumulative effluent volume was created in the R statistical software environment (R Development Core Team 2015).

2.5 Statistical analysis

The mean concentrations and removal efficiencies of the measured pollutants were calculated for each week of biological regeneration testing as well as the total dataset. Differences in pollutant concentrations, sorption, and removal between the planted and the unplanted sides were evaluated with one-way analysis of variance (ANOVA). The analyses were performed in the R statistical software environment (R Development Core Team 2015).

3 Results and discussion

3.1 Biological regeneration

The rhizofiltration system received no wastewater for 5 months prior to testing the regeneration capacity of the filter media. It is therefore likely that pollutant concentrations in the filter medium were low at the beginning of experimentation. This development made it possible to determine the biological regeneration of the rhizofilter anew.

The planted and the unplanted sides of the rhizofilter showed NH_4^+ removal efficiencies of 17.7 ± 6.3 % and 24.9 ± 3.4 % respectively (Table 2.1). The effluent concentrations generally followed variations in the influent except for the samples taken during the sixth week where NH_4^+ removal (~60 %) was highest (Figure 2.1). On average approximately 10 mg/L NH_4^+ was removed by each side. Furthermore, NO_3^- measurements indicated that nitrification took place in both sides, since higher NO_3^- concentrations were found in the effluent, compared to the influent, especially during the first six weeks (Figure 2.2; Table 2.1). The highest NO_3^- concentration was observed during the sixth week, which corresponds to the highest percentage NH_4^+ removal recorded during the sampling period. The NO_3^- concentrations were similar for the planted and unplanted sides with a mean increase of 46.1 ± 17.3 % and 39.1 ± 18.3 % respectively (Table 2.1). The lack of an

increase in NO_3^- concentrations during the last four weeks of testing (Figure 2.2) may indicate that coupled nitrification-denitrification took place since NH_4^+ was still being removed during this period (Figure 2.1). Alternatively, the lower influent COD concentrations (~650 mg/L) during the last three weeks (Figure 2.3) may have limited the carbon supply for aerobic heterotrophic respiration in the filter medium. Subsequently lower amounts of CO_2 were probably produced, which may have led to reduced nitrification.

The efficiency of the rhizofilter in removing NH_4^+ and COD (~14.8 % for both the planted and unplanted side; Table 2.1) from the influent may be due to sufficient air flow through the filter media facilitated by the large rocks underneath the sand layer and the intermittent feeding mode (Brix and Arias, 2005). This creates favorable conditions for the oxidation of NH_4^+ to NO_3^- via nitrite (NO_2^-) by chemolithoautotrophic bacteria capable of using inorganic nitrogen as an energy source (Abou-Elela et al., 2013; Caselles-Osorio et al., 2011; Gregory et al., 2010; Wiessner et al., 2005). The continuous removal of NH_4^+ and COD from the formulated influent indicated that the filter medium was regenerated in the seven-days between wastewater additions (Figures 2.1 and 2.3).

Unlike the previous monthly performance results (Wilsenach et al., 2014), higher mean PO_4^{3-} removal was observed in this study with 42.6 ± 10.0 % and 43.7 ± 12.8 % for the planted and unplanted sides respectively (Table 2.1, Figure 2.4). The best removal efficiencies were at higher influent concentrations (Figure 2.4). This was also the case for the removal of SO_4^{2-} (Figure 2.5) by the planted (54.9 ± 7.6 %) and unplanted (51.3 ± 8.3 %) sides. The SO_4^{2-} and the suspended solids concentrations differed significantly (ANOVA; $p < 0.05$) from the influent values (Table 2.1). The effluent concentrations of the suspended solids were relatively constant (Figure 2.6) ranging between 26.5 and 66 mg/L

with a removal efficiency of 49.9 ± 6.4 % (planted) and 53.3 ± 6.7 % (unplanted). The highest removal was observed during the final week when a very high influent suspended solids concentration of 181 mg/L coupled with lower effluent concentrations resulted in a removal efficiency of ~90 % (Figure 2.6). Higher suspended organic and colloidal pollutant concentrations in the formulated influent could form a crust on top of the filter media or reduce the pore volume in the filter media, creating an extra barrier in which pollutants become trapped (Langergraber et al., 2003; Vymazal, 2011). This results in increased removal of pollutants as wastewater percolates through the filter media. However, the filter medium may clog if the particles trapped in the medium are not removed through the biological degradation processes.

Effluent values of the microbiological pollution indicators (faecal coliforms, *Salmonella/Shigella* and coliphages) were strongly dependent on the influent values throughout the sampling period (Figures 2.7, 2.8, and 2.9). Removal efficiencies were low (< 7 %) for all three microbiological parameters with less than a log reduction in concentration (Table 2.1). The removal efficiency of the indicator bacteria was higher on the unplanted side than the planted side. This may be due to the slower percolation rate of the unplanted side allowing more bacterial cells to be adsorbed onto the filter medium and the suspended organic material trapped in the filter medium (Mburu et al., 2008; Okurut, 2000)

Rhizofiltration relies predominantly on the removal of pollutants via physical processes such as sorption, while water percolates through the system (Mclean et al., 2006). Subsequently, pollutants trapped in the filter media are removed through biological processes (Carleton et al., 2000; Dushenkov et al., 1997; Picard et al., 2005). We have shown that the pollutant removal capacity of the rhizofilter could be restored within a week

for some pollutants such as NH_4^+. However, the amount of pollutants that can be removed by the rhizofilter during a storm event needs to be estimated to determine the operational parameters wherein the rhizofilter would be effective. To obtain an estimation of the capacity of the filter to remove pollutants during the relative short period of a storm, the sorption capacity of the rhizofilter was determined.

3.2 Sorption capacity

The sorption capacity of the rhizofiltration system, with regard to biological and chemical pollutants was determined both for the planted and unplanted sides. The breakthrough curves (Figure 2.10) show the loading and removal behaviour of the pollutants and are each expressed as a normalized concentration defined as the ratio of effluent concentration (C) to influent concentration (C_o) as a function of the cumulative effluent volume (L). Sigmoidal profiles were observed in the breakthrough curves of NH_4^+, faecal coliforms and *Salmonella/Shigella* values (Figure 2.10). This is consistent with previous studies, which have found that as pollutants are released on to a filtration device; the upper zone of the filter medium becomes saturated causing a systematic decrease in sorption capacity (Aksu et al., 2002; Aksu & Gönen, 2004; McGinley et al., 1996; Rojas-Mayorga et al., 2015; Wolborska, 1999). This in turn creates an instantaneous jump in pollutant concentrations found in the effluent. The shape of breakthrough curves depends on bed geometry, operating conditions, transport properties, heat effects, and equilibrium adsorption isotherms (Park and Knaebel, 1992; Zhang et al., 2006). Factors that could have contributed to the unusual breakthrough curves observed in this study for some of the measured pollutants (Figure 2.10) include the three different filter layers, plant roots, and the build-up of solids forming a sludge layer on the filter sand (Figure 2.10) (Allaire-Leung et al., 2000; Rojas-Mayorga et al., 2015). The slope of the breakthrough curve is an indication of the saturation rate of the filter medium for a particular pollutant (Chu, 2004).

The planted and unplanted sides of the biofilter, removed similar amounts of NH_4^+, but the unplanted side proved to be more efficient, with a greater overall percentage removal (Figure 2.10; Table 2.2). This was also the case for NO_3^- levels. However, there was a constant decrease in the C/C_o ratio of NO_3^-, indicating a buildup of NO_3^- on both sides of the rhizofilter between performance testing events. Previous performance testing results showed very low NO_3^- concentrations in the effluents of both the control and experimental sides when sampled after a percolation time of 45 minutes (Wilsenach et al., 2014). However, the current results show that nitrification occurred between performance testing events which resulted in high initial NO_3^- concentrations in the effluent, followed by a systematic decrease of NO_3^- concentrations in the effluent during sorption testing (Abou-Elela et al., 2013; Caselles-Osorio et al., 2011).

A decrease in the PO_4^{3-} C/C_o values was observed on both sides of the filter after the initial load of formulated influent, where after it increased slightly on the planted side but never reached equilibrium (Figure 2.10). The relatively constant C/C_o value (below 0.5) on the planted side may be an indication that both sorption and desorption processes occurred, but with net sorption. For the unplanted control side, the PO_4^{3-} C/C_o values reached equilibrium after approximately 3000 L of wastewater percolated through the filter. The initial decrease in C/C_o value could be caused by residual PO_4^{3-} present in the filter media from a previous flush event, after the system has reached maximum sorption capacity. According to Arias and co-workers (2001), one way to ensure sustained PO_4^{3-} removal is by harvesting the plants growing in these systems. Another approach is to use a filter medium with an increased phosphate sorption capacity (Caselles-Osorio et al., 2011; Prochaska et al., 2007; Vymazal, 2011). The mean removal percentage for the planted and unplanted sides of the filter was 66.6 ± 7.8 % and 67.6 ± 10.7 % respectively. This indicates that the filter media in the rhizofilter is sufficient for phosphate removal however

due to possible phosphate leakage from decaying plant material there was no significant difference observed in PO_4^{3-} removal, between the two sides (Table 2.2). Other factors that may have influenced the removal of PO_4^{3-} may include the chemical properties of the wastewater such aluminium, iron and calcium concentrations as wells as pH. The pH of the formulated influent used during these experiments was constant at about 6.1 (data not shown). The slightly acidic nature of the formulated influent may cause PO_4^{3-} to bind with positively charge metals forming insoluble compounds that are not readily available for uptake by plants (Bieleski 1973).

The SO_4^{2-} in the wastewater was adsorbed by both sides of the filtration system, however the unplanted control side showed a higher percentage removal of approximately 75 % compared to the 62 % of the planted side. Neither side appeared to reach equilibrium during the 45 min test period, which could indicate that the system can accommodate larger amounts of SO_4^{2-}. The removal of inorganic sulphur is associated with microbial biofilm activity where SO_4^{2-} is adsorbed to the biofilm surface and possibly reduced to sulphide within the inner anaerobic layers of the biofilm (Vymazal and Kröpfelová, 2009). This mechanism was considered to be one of the most dominant processes involved in sulphur removal from constructed wetlands (Bernardo et al., 2004). However, since the rhizofilter is predominantly aerobic, with the helophytes contributing to the oxygen concentration in the filter bed, anaerobic reduction of SO_4^{2-} may have been inhibited on the planted side of the rhizofilter (Brix and Arias, 2005; Rousseau et al., 2004).

During sorption testing about 2 400 g of the COD in the influent was removed on each side of the rhizofilter (Table 2.2). This relates to the removal of approximately 37 % and 41 % of the COD that percolated through the planted and unplanted sides respectively. There was

no significant difference between these two sides. The combined effect of physical and microbial mechanisms influences the removal of COD in vertical flow wetlands (Abou-Elela et al., 2013; Zhang et al., 2006). The sedimentation of organic matter, especially in the sand layer of the rhizofilter, can increase the contact time with microbial communities present in the media. This allows for increased biodegradation of these organic solids between urban runoff events. However, the lack of significant difference between the planted and the unplanted sides, in terms of COD removal, can be due to the short hydraulic retention time of the formulated influent in the rhizofilter. Thus, causing limited time for biological mechanisms to remove COD since it is known that biological activity is closely linked to the growth rate of the organisms involved (Toet et al., 2005). This may be the case for all the nutrient removal processes associated with the rhizofilter.

The continuous decrease in the suspended solids C/C_o ratios on both sides of the rhizofilter could indicate a possible buildup of organic material on top of the filter media, creating an extra barrier in which suspended solids were trapped. Furthermore, the unplanted control side appeared to reach equilibrium after 2000 L of wastewater percolated through the system (Figure 2.10). In contrast, the suspended solids C/C_o ratio on the planted side decreased steadily, indicating gradual clogging of the system. The breakthrough curves of the suspended solids concentrations could be used as an indication of how rapidly the filter may clog. One of the major problems associated with vertical wetlands is substrate clogging (Langergraber et al., 2003; Vymazal, 2011), which is caused by a number of factors that include the size of the biofilter, distribution and compaction of the media, suspended solids loading concentration, chemical precipitation as well as the growth of plant-rhizomes and roots (Langergraber et al., 2003). This clogging of the filter media could result in reduced oxygen diffusion and ultimately in decreased rhizofiltration performance with regard to nutrient removal (Zhu et al., 2012).

The three microbiological parameters showed similar breakthrough curves during sorption testing, with initial C/C_o ratio values for all three being approximately 0.8, indicating that these indicator organisms possibly persist in the filter bed. Equilibrium for the coliphages was reached after the addition of just only 1000 L of formulated influent. Moreover, equilibrium was reached at 2000 L for both the faecal coliforms and *Salmonella/Shigella,* with overall pollutant removal being more efficient on the planted side of the biofilter than on the unplanted side. Some of the mechanisms commonly associated with the removal of these indicator organisms in the vertical flow wetland systems include antimicrobial activity from root exudates, such as gallic and tannic acids, biofilm retention, as well as oxidation, which could explain the increased removal efficiency of all three microbiological parameters on the planted side of the biofilter (Decamp & Warren, 2000; Vacca et al., 2005).

Generally, sorption testing revealed that the unplanted side was superior or similar to the planted side of the rhizofilter with regard to pollutant removal (Table 2.2), this is in contrast to comparative studies conducted by others on wetlands who found indications of an overall improvement in pollutant removal performance in the presence of vegetation in the filter bed (Abou-Elela et al., 2012). These authors attributed the improved pollutant removal performance to wetland plants releasing oxygen into the rhizosphere, creating a spatial and temporal micro-scale gradient of oxygen concentrations and redox states close to root surfaces, potentially increasing the oxidation of nutrients (Abou-Elela et al., 2012; Caselles-Osorio et al., 2011; Gregory et al., 2010; Wiessner et al., 2005). However, the influence of plant oxygen released into the rhizofilter systems may be negligible, due to high amounts of oxygen already permeating through the filter media, caused by intermittent batch feeding and the vertical flow of fed influent (Faulwetter et al., 2009;

Piccard et al., 2005). Interestingly, the planted rhizofilter showed a higher flow rate than the unplanted filter. This phenomenon may be ascribed to the rhizomes and roots of wetland plants creating channels in the filter media, which facilitates water flow through the planted filter bed (Abou-Elela et al., 2013). Thus, the increased removal efficiency of the unplanted side of the filter recorded for some of the pollutants (Table 2.2) could possibly be explained by the increased density of the filter media and the concomitant slower percolation rate. The higher effluent volume and percolation rate on the planted side of the filter, could possibly have led to less contact time of pollutants with the filter media reducing the removal efficiency.

4 Conclusions

In this study we determined the overall performance of a pilot scale rhizofiltration system, with regard to the bioregeneration and sorption capacity capabilities of the rhizofilter. Performance tests showed that the filter medium could be partially regenerated within a week. Similarities in sorption capacity performance of the unplanted and planted sides of the rhizofilter may be due to similar adsorption-desorption kinetics within the filter medium. We also observed that the density of the filter media was consequential to the overall performance of the rhizofilter, in terms of sorption capacity. Although these physical properties play a significant role in pollutant removal from urban runoff, previous studies performed on numerous types of vegetated biofilters, revealed that the microbial communities present inside the filter media are strong determinants of performance. Research on the microbial communities occurring in the filter media and their response to pollutant loads, as presented in the following chapter, may provide more insight on the mechanisms involved in pollutant removal.

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Figures and tables

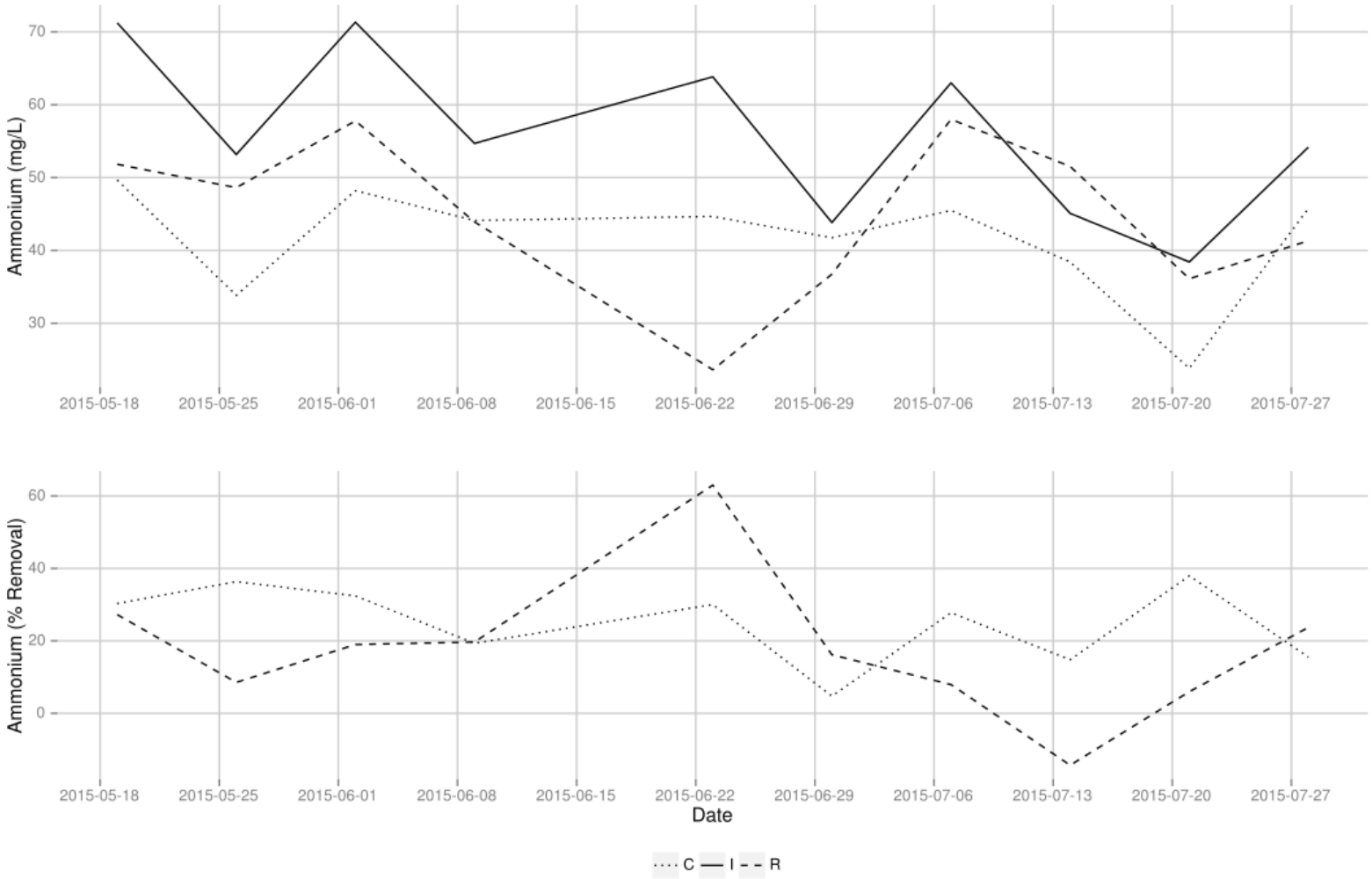

Figure 2.1: Ammonium concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

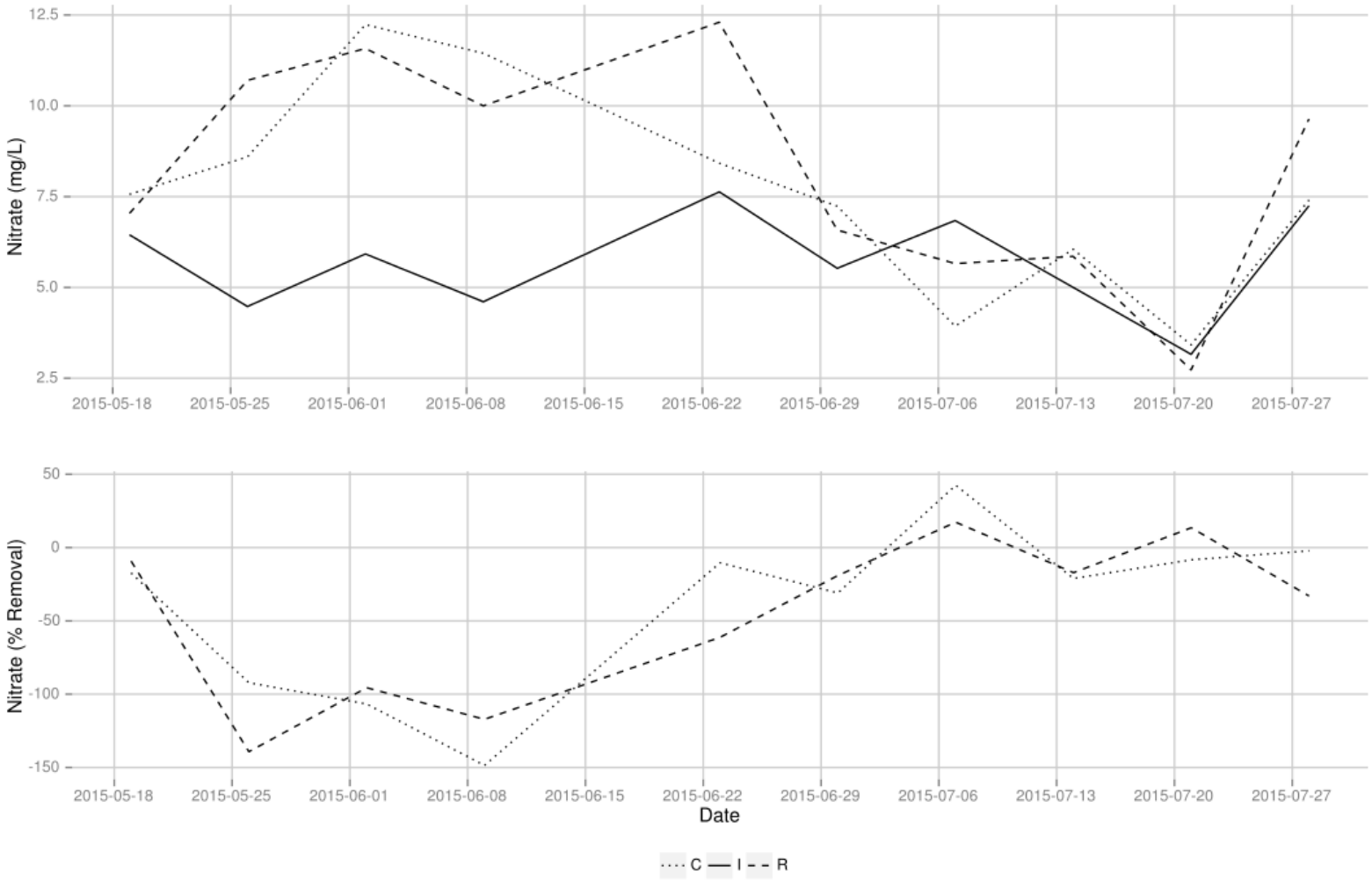

Figure 2.2: Nitrate concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

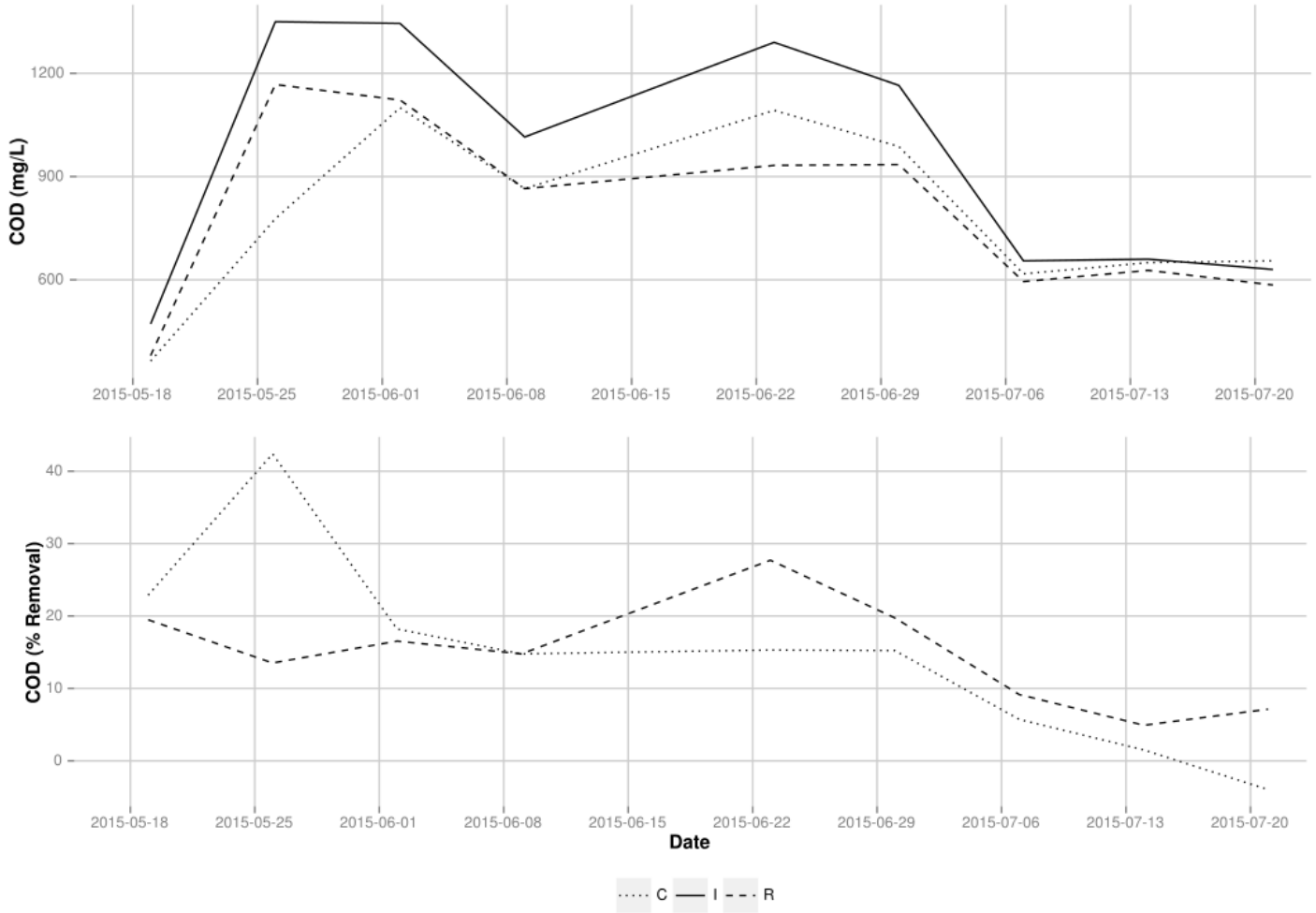

Figure 2.3: Chemical oxygen demand (COD) concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

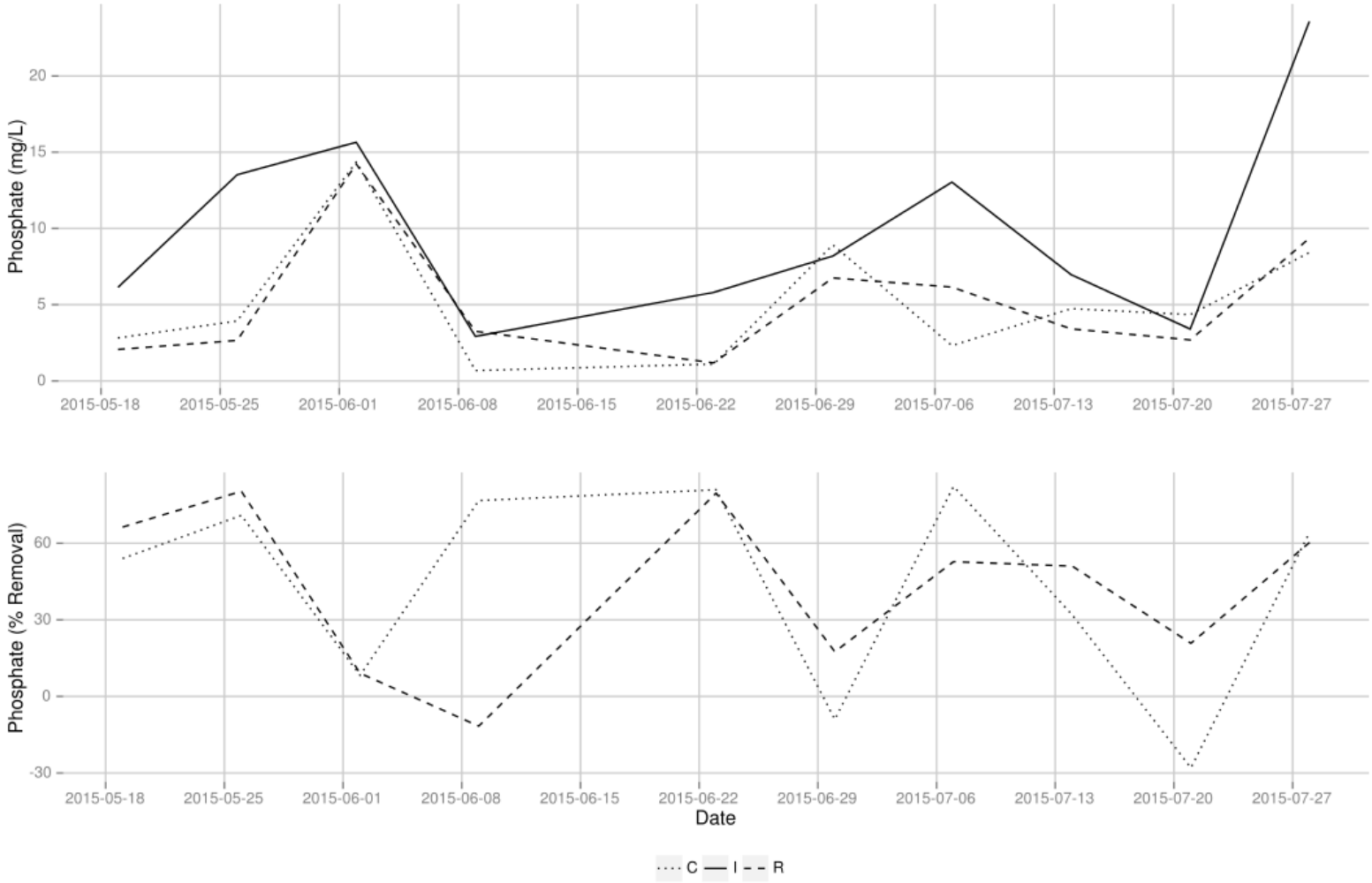

Figure 2.4: Phosphate concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

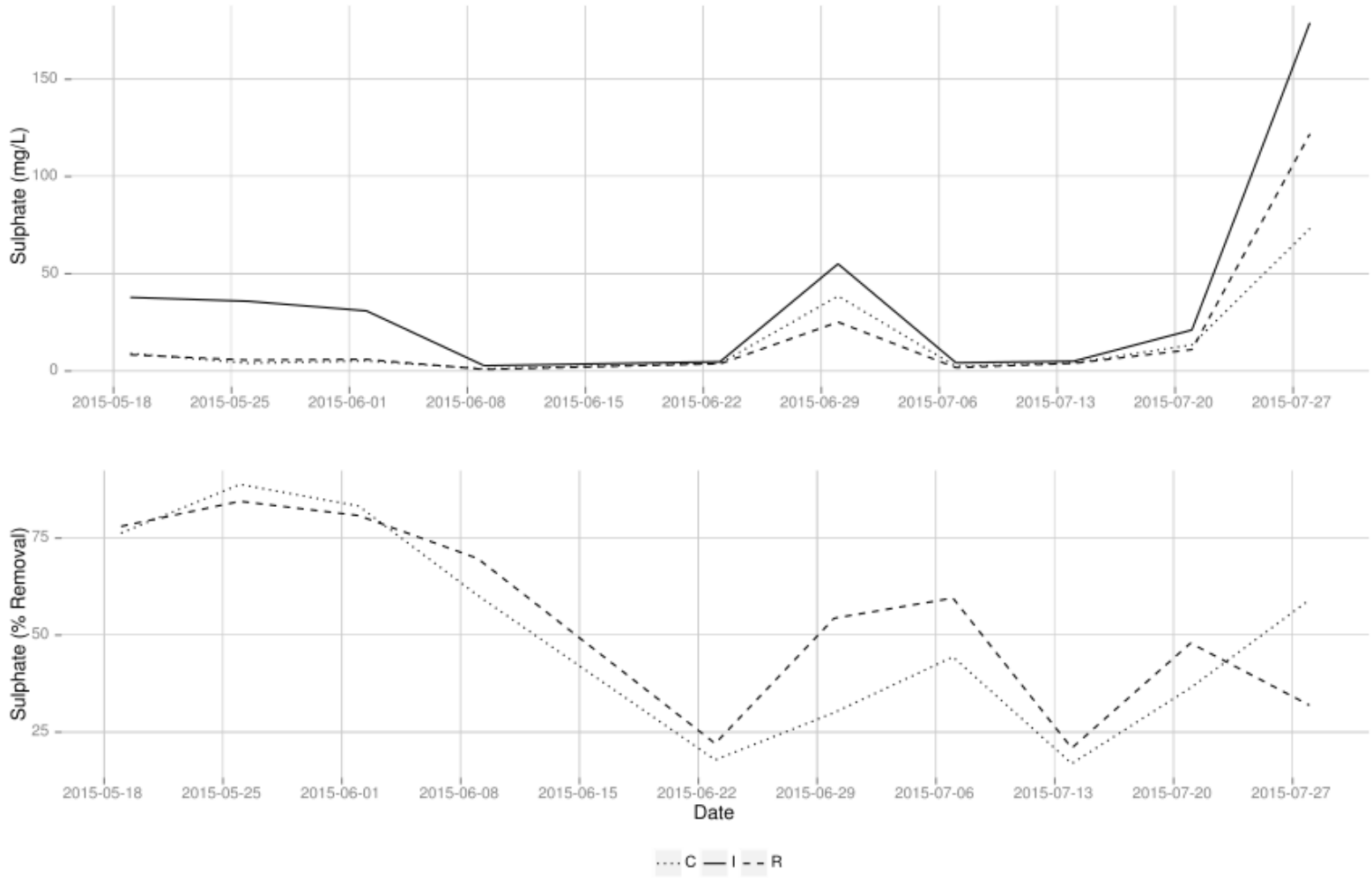

Figure 2.5: Sulphate concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

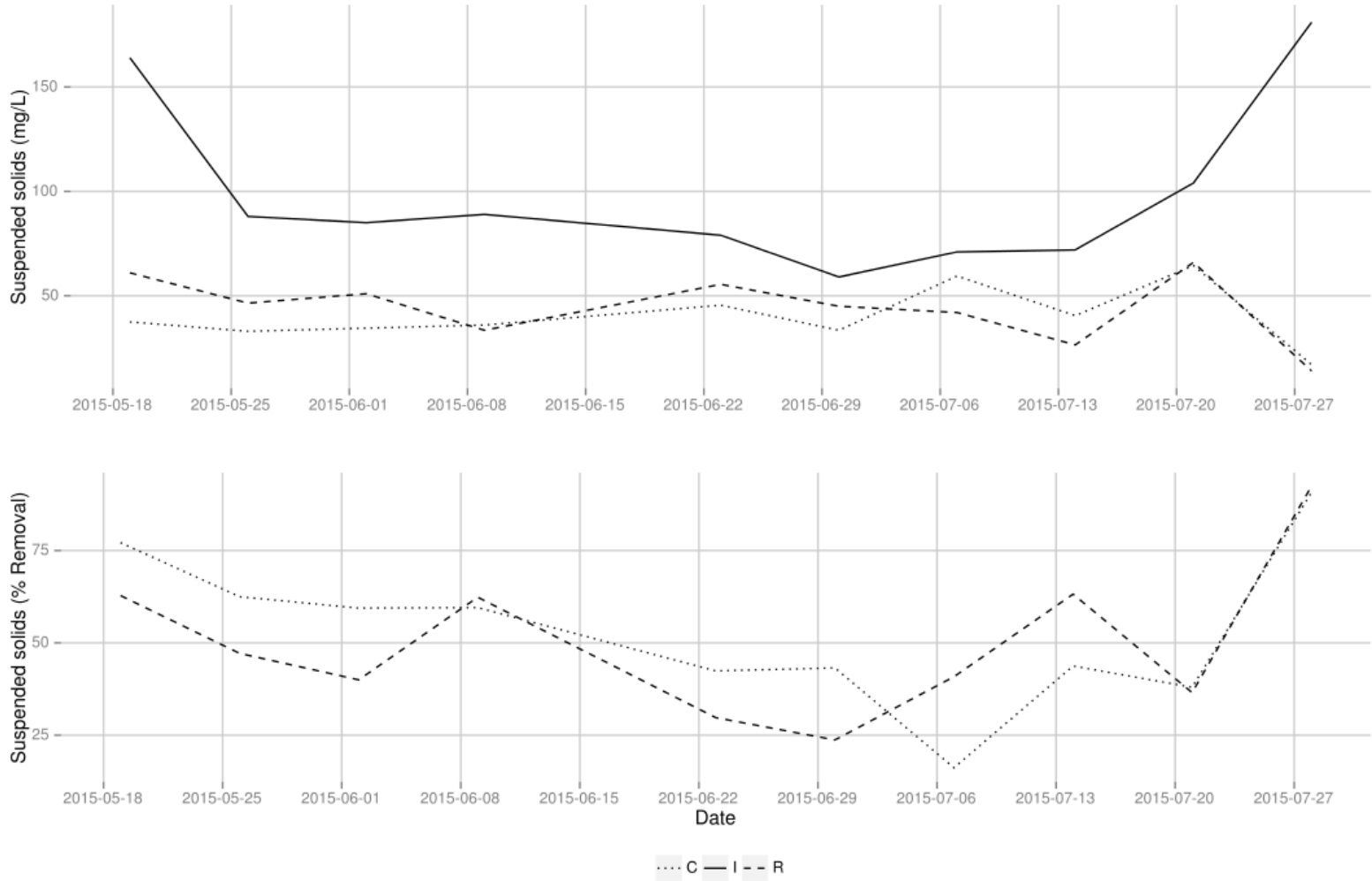

Figure 2.6: Suspended solids concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

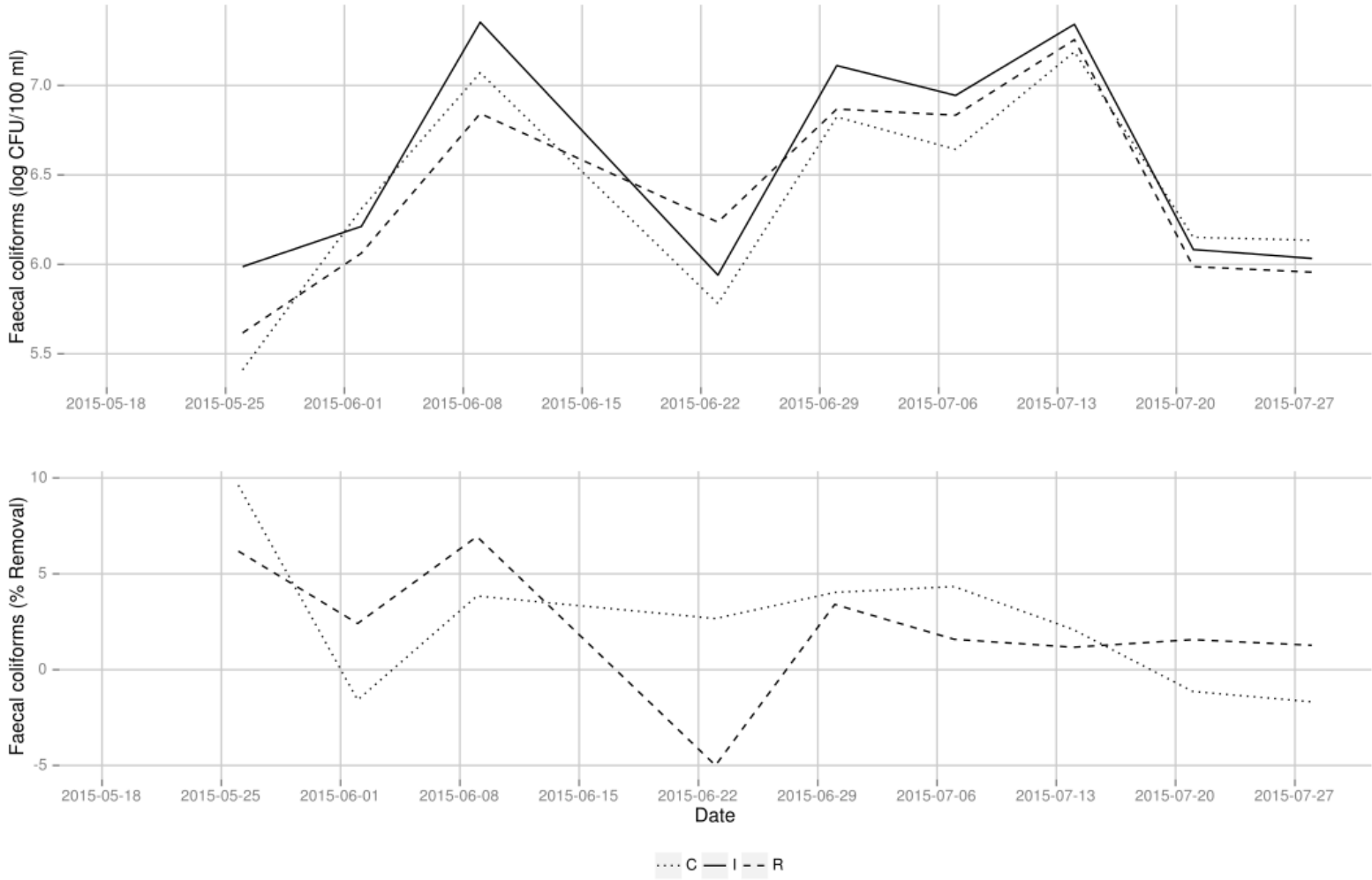

Figure 2.7: Faecal coliform concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

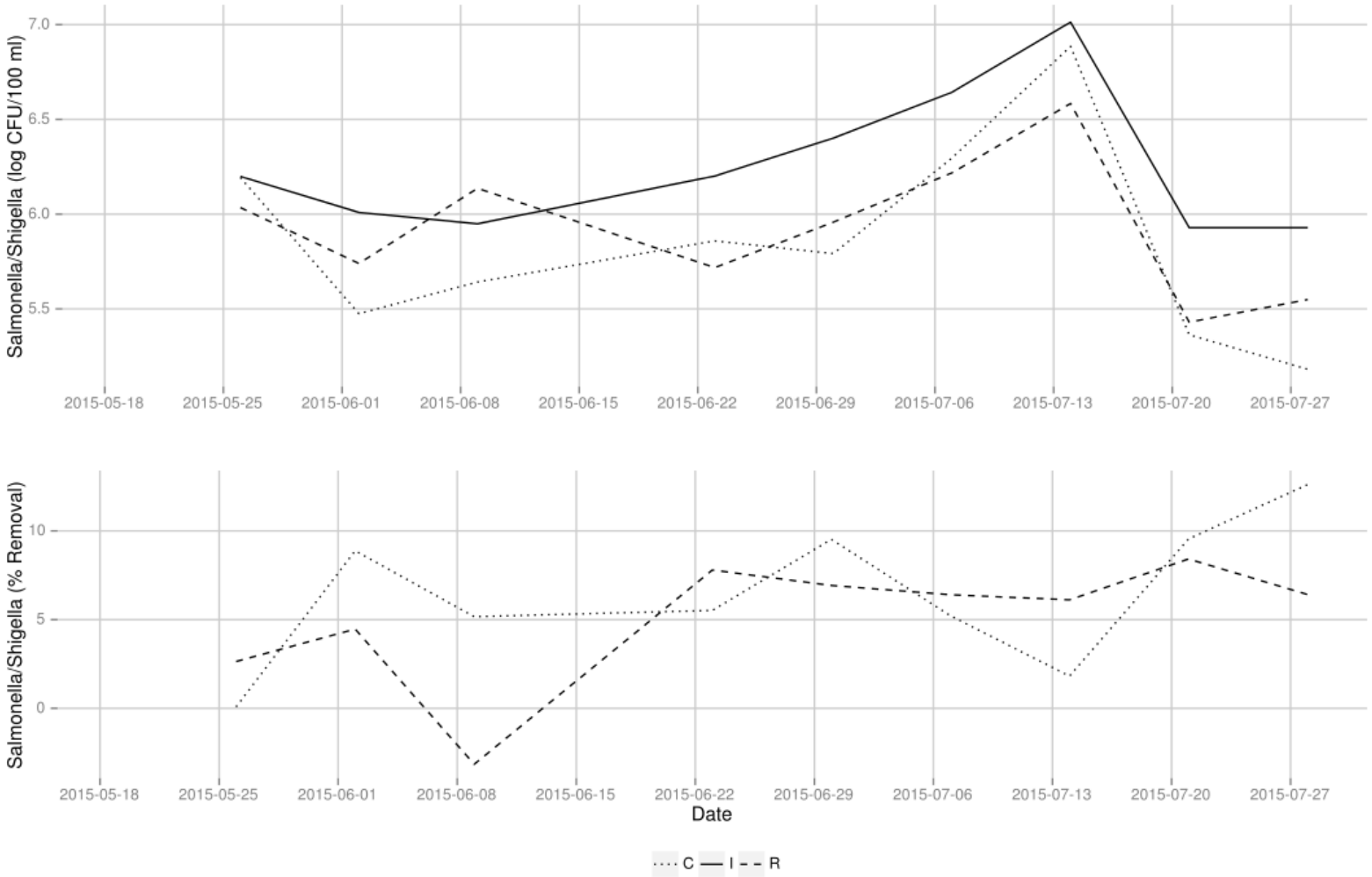

Figure 2.8: *Salmonella/Shigella* concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

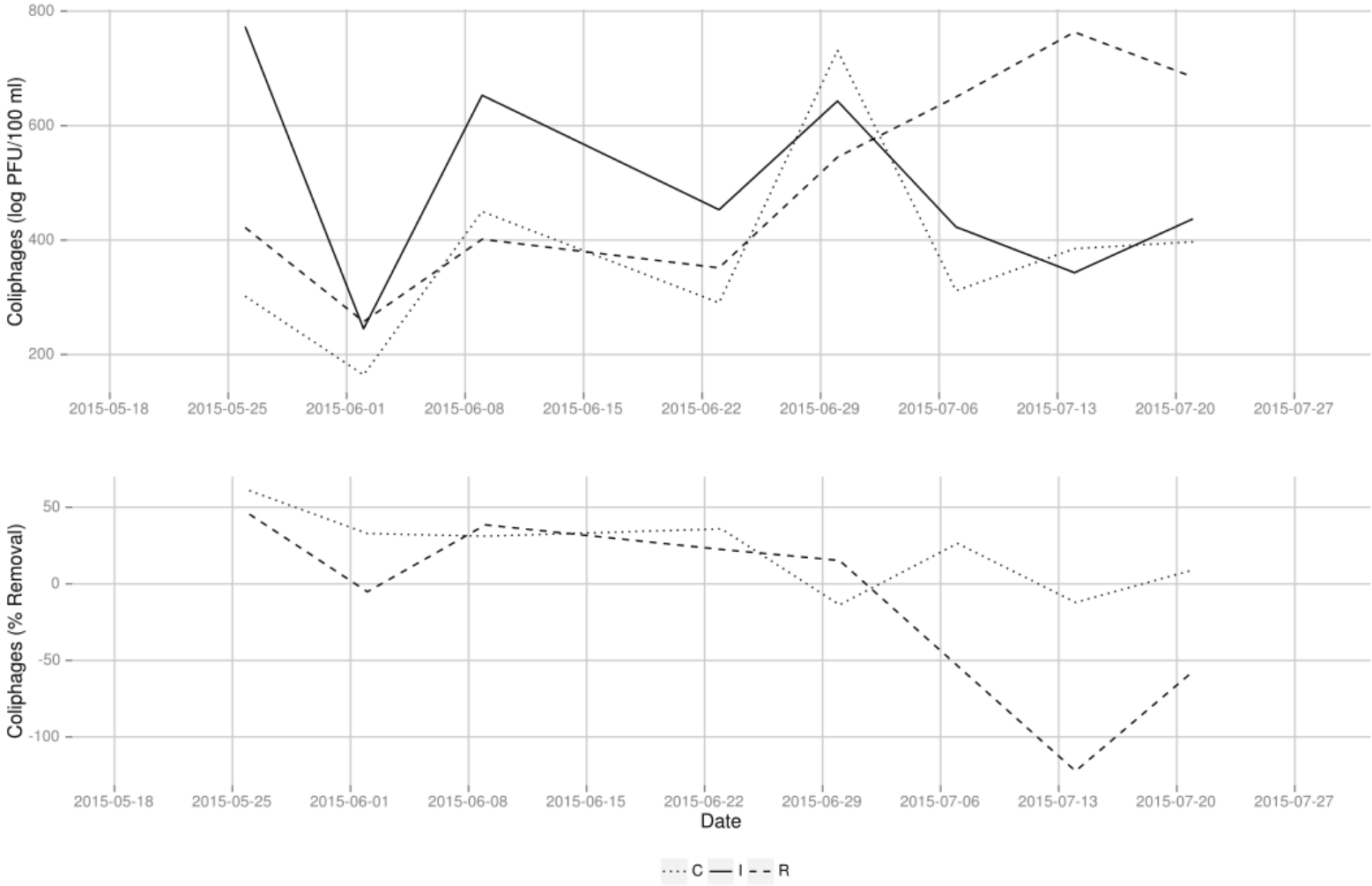

Figure 2.9: Coliphage concentrations and removal on the planted (R; experiment) and the unplanted side (C; control) of the rhizofiltration system after simulated urban effluent percolated through it for 45 minutes. Values are the mean of effluent samples collected from two wells on both the unplanted and planted sides. Influent (I) values are from one sample collected each week.

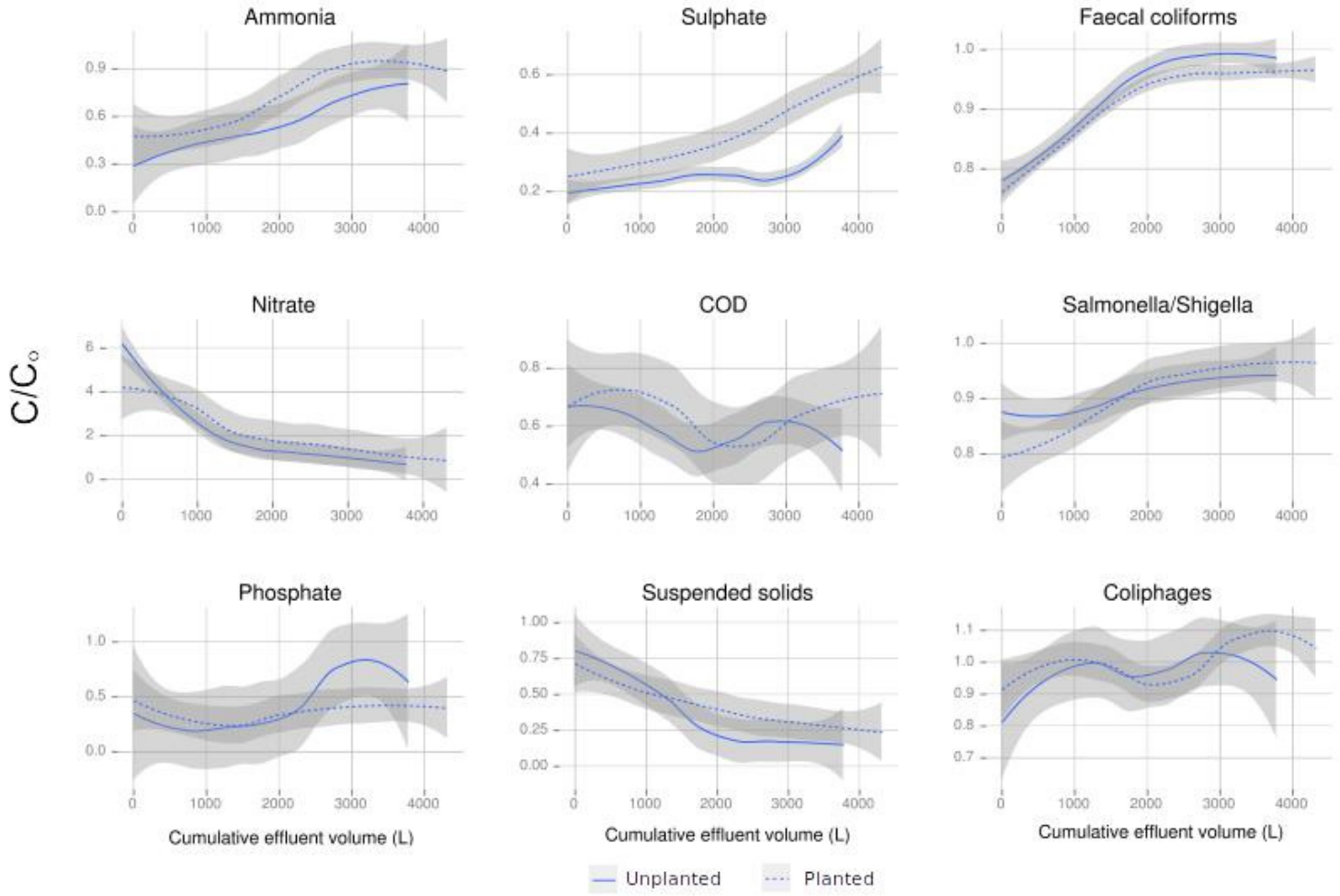

Figure 2.10: Breakthrough curves of the unplanted (control) and planted (experiment) sides of the rhizofilter with regards to the measured chemical and microbiological pollutants. Y-axis values are the effluent/influent ratios at 3 minute time intervals for a total duration of 48 minutes calculated from triplicate samples collected on three separate occasions. Curves are LOESS smoothed regressions and the shaded areas indicate a 95 % confidence interval.

Table 2.1: Summary of means of microbiological, physical and chemical data from weekly sampling events.

Parameter	Influent	Effluent		Efficiency (%)	
		Planted (experiment)	Unplanted (control)	Planted (experiment)	Unplanted (control)
Faecal Coliforms ♦	6.6 ± 0.2 (9)	6.4 ± 0.2 (9)	6.3 ± 0.2 (9)	2.2 ± 1 (9)	5.1 ± 1 (9)
Salmonella/Shigella ♦	6.3 ± 0.1 (9)	5.9 ± 0.1 (9)	5.8 ± 0.2 (9)	5.1 ± 1 (9)	6.5 ± 1 (9)
Coliphages ♦	4.7 ± 0.1 (8)	4.6 ± 0.04 (8)	4.5 ± 0.1 (8)	1.0 ± 1 (8)	3.0 ± 1 (8)
Ammonia▪	55.8 ± 3.6 (10)	44.9 ± 3.4 (10)	45.7 ± 2.5* (10)	17.7 ± 6.3 (10)	24.9 ± 3.4 (10)
Nitrate▪	5.6 ± 0.4 (10)	8.3 ± 0.9 (10)	7.6 ± 0.8 (10)	-46.1± 17.3 (10)	-39.1 ± 18.3 (10)
Orthophosphate▪	9.9 ± 2.0 (10)	5.2 ± 1.3 (10)	5.1 ± 1.3 (10)	42.6 ± 10.0 (10)	43.7 ± 12.8 (10)
Sulphate▪	37.6 ± 16.7 (10)	18.8± 11.7 (10)	15.48 ± 7.3 (10)	54.9 ± 7.6 (10)	51.3 ± 8.3 (10)
COD▪	954 ± 117 (9)	801 ± 89 (9)	790 ± 82 (9)	14.8 ± 0.02 (9)	14.7 ± 0.04 (9)
Suspended Solids▪	99.2 ± 12.87 (10)	44.1 ± 5.0** (10)	40.2 ± 4.3 ** (10)	49.9 ± 6.4 (10)	53.3 ± 6.7 (10)

♦Log Colony Forming Units (CFUs) / 100 mL; ▪ mg/L
ANOVA significant difference p-values: < 0.5 = *, <0.1= **, * significantly different from influent. Numbers in parenthesis = N data points.

Table 2.2: Total quantity of pollutants adsorbed (qtotal) and removed determined by calculating the area under the breakthrough curves and comparing it to the total pollutant load (m_{total}) that percolated through the filter medium on the planted (experiment) and unplanted (control) sides of the rhizofilter.

	Mtotal		qtotal		Removal (%)	
	Planted	**Unplanted**	**Planted**	**Unplanted**	**Planted**	**Unplanted**
Ammonia (g)	235.7±3.9	206.2±3.4	67.2±31.4	91.4±20.4	28.5±13.3	44.3±9.9*
Nitrate (g)	26.8±1.7	23.4±1.5	1.1±29.4	1.4±0.3	4.1±1.1	5.8±1.4*
Phosphate (g)	57.4±20.8	50.2±18.2	39.5±14.7	36.9±15.7	66.6±7.8	67.6±10.7
Sulphate (g)	789.9±77.8	691.2±68.1	477.6±90.9	519.5±53.4	62±4.4	75.1±0.58
COD (g)	6576.5	5754.4	2453.4	2401.2	37.3	41.7
Suspended solids (g)	685±81.5	599.4±71.3	427.6±142.1	404±130.5	59.9±13	64.6±13.2*
Faecal coliforms (log CFU)	9.52±0.02	9.46±0.02	8.81±0.02	8.65±0.02	19.68±0.2	15.33±0.05
Salmonella/Shigella (log CFU)	9.08±0.3	9.02±0.3	8.36±0.3	8.36±0.2	19.6±4.29	22.7±4.3
Coliphages (log PFU)	8.4	8.3	7.7	7.75	20.7	25.7

*Significantly (ANOVA $p < 0.05$) different from the planted side.

Chapter 3

Dynamics of a simulated riparian microbiome treating severely polluted urban effluent

1 Introduction

The global problem of urban runoff increasingly threatens the quality of fresh and coastal waters (Fletcher et al., 2008; Granato, 2014; Jia et al., 2015). Urban areas are mostly covered with impermeable surfaces on which various pollutants accumulate (Göbel et al., 2007). Precipitation events flush these pollutants into natural water courses through engineered or semi-natural channels. This problem is often exacerbated by inadequate or malfunctioning waste and stormwater infrastructure (Dungeni et al., 2010; Samie et al., 2009). Consequently, the pollutant loads in the urban effluent of underdeveloped areas are often comparable to municipal wastewaters (Paulse et al., 2009). As a result, pollutants in urban runoff may not only include nutrients and anthropogenic chemicals, but also human pathogens and parasites (Göbel et al., 2007; Granato, 2014). Apart from the associated potential human health risks, natural ecosystem function is often disturbed by eutrophication caused by excessive levels of nitrogen and phosphorus (De-Bashan and Bashan, 2004).

To address the above-mentioned pollution, low impact development best management practices (LID-BMPs) are increasingly being employed on a global level (Dietz, 2007; Liu et al., 2015). These practices are small-scale and localized source control measures, designed to replicate a location's natural properties with processes such as infiltration, evaporation and filtration. Pollutants trapped in these systems are removed through complex physical, chemical and biological mechanisms (Faulwetter et al., 2009). The latter includes nutrient removal by microbial communities. Additionally, vegetation is believed to create a more favourable environment for treatment processes to occur (Truu et al., 2009; Ulrich et al., 2005).

Few investigations aimed at measuring BMP performance consider the underlying

microbial ecology (Faulwetter et al., 2009; Truu et al., 2009). As a result, there is a lack of species specific data to develop conceptual frameworks about the dynamics of microbial community structure, its relationship to removal processes, and operational parameters for these practices. It is often assumed that microbial processes are important contributors to pollutant removal, and indeed BMPs are usually effective against the most common pollutant indicators (Granato, 2014). However, we may underestimate the potentially negative contribution of microbial processes, such as the generation of harmful greenhouse gasses or the retention of human pathogens in these systems (Boutilier et al., 2009). Although the positive effects of BMPs in pollution removal may outweigh the negative effects, an improved understanding of the related microbial ecology may allow for better informed BMP designs. Aspects of microbial communities that could affect BMP performance include structure, stability, functional redundancy and diversity (Hu et al., 2012; Truu et al., 2009).

The microbial diversity of wastewater ecosystems can be characterized and quantified at a very high resolution using high throughput sequencing (HTS) of taxonomically informative gene loci (Ju and Zhang, 2014). However, to adequately estimate performance characteristics of wastewater treatment systems, both the biological and coupled chemical kinetics need to be measured (Van Loosdrecht et al., 2008). Thus, it is essential to link DNA sequence abundance to chemical indicators of the biomass or growth rate of microbial assemblages. Previously, phospholipid fatty acid (PLFA) analysis was successfully applied to monitor changes in microbial biomass, and activity (Ibekwe and Kennedy, 1999; Watzinger et al., 2008; Zhao et al., 2012). Combining HTS and PLFA analysis may therefore provide valuable insights into the relationship between bacterial diversity, biomass and function in both engineered and natural ecosystems.

In this study, we investigated the microbiome of a pilot scale LID-BMP which was designed to mimic riparian ecology, with the goal of rapidly filtering large volumes of urban effluent before it enters rivers. We aimed to characterize the microbial community structure and temporal stability of this system, and garnered novel information about the bacterial assemblages and the selective role of commonly occurring aquatic plants. We also present the first study linking microbial DNA sequence data with PLFA biomass markers and constituents of pollutant loads expected to occur in urban runoff. In future, this information may be valuable to construct mechanistic models of pollutant treatment mechanisms in engineered and natural riparian zones.

2 Materials and methods

2.1 Experimental design

A pilot scale LID-BMP that incorporates the concept of rhizofiltration (Dushenkov et al., 1997; Cardinali et al., 2014; Lee and Yang, 2010; Veselý et al., 2011) was constructed at the Stellenbosch wastewater treatment works (Figure 1.1), Western Cape, South Africa (33,94335S-18,82425E). The region has a Mediterranean climate characterized by hot, dry summers and cool, moist winters (Di Castri, 1991). The LID-BMP (henceforth referred to as the rhizofilter) is an aboveground concrete structure 7.5 m long, with a depth of 1 m and width of 3 m. To test the hypothesis that plants select for specific microbial populations, the rhizofilter was divided lengthwise using a wire mesh covered by high density polyethylene sheets, to create two separate basins. Both basins were filled with a filter medium, which consisted of three ca. 3 m^3 layers of rocks and sand, ranging from coarse rocks (100 -120 mm diameter) at the bottom, to crushed rocks (19 - 25 mm diameter) in the middle, and a top layer of coarse river sand. One of the basins was evenly planted (the experimental side) with two helophyte species, namely *Typha capensis* (Rohrb.) and *Phragmites australis* (Cav.; Steud.), while the control side remained unplanted.

The rhizofilter was in operation for 30 months prior to the current study. To evaluate the rhizofilter microbial community's response to wastewater additions, the experimental procedure outlined below was repeated over a period of three months during the winter, i.e. April, May and July 2014. Settled municipal wastewater was pumped into a 10 000 L tank and allowed to settle overnight. The first 2 000 L of water was flushed to remove excessive solids. The remainder (±8 000 L) was distributed equally between the planted and unplanted sides of the system through 2 pairs of overhead valves at a rate of 7 $L.s^{-1}$. This formulated influent percolated vertically through the system for 45 minutes.

2.2 Sampling

Water samples (1 L) were collected from the influent and effluent on both the planted and unplanted sides and the temperatures measured immediately. A soil corer with a diameter of 10 cm was used to collect sand samples to a depth of 15 cm immediately before, as well as one, 24, 72 and 168 h after the addition of wastewater. The soil corer was sterilized between sites. Three replicate samples (±250 g wet weight) each consisting of three pooled sand cores were collected along the length of the control side. Replicate rhizosphere and bulk sand samples were collected from the planted side by removing five mature *T. capensis* plants (the dominant species) along the length of the filter. The plant rhizomes were vigorously shaken in a plastic bag to collect the bulk sand fraction (±300 g wet weight each). The rhizomes were subsequently put in separate sealable plastic bags which were transported on ice to the laboratory. The rhizosphere sand fraction of each plant (±150 g wet weight), consisting of sand that tightly adhered to the roots, was collected using a sterile forceps. All replicate samples were then pooled and homogenized by sieving the samples (2.8 mm mesh) which simultaneously removed debris. In total 45 composite samples were collected, one sample per sand fraction at each time interval over three months. These samples were sub-fractionated for PLFA, chemical, and HTS

analyses, and stored at -20 °C until extraction.

2.3 Chemical and microbiological analyses of water and sand

The purpose of the rhizofilter was to remove nutrients that could cause eutrophication. For effective removal we hypothesized that changes in the bacterial communities in the filter media would strongly depend on the nutrient dynamics, and vice versa. The total carbon (C) and nitrogen (N) content of the sand fractions were determined using a Leco CHN-628 element analyser (LECO Corporation, MI, USA). Nitrate (NO_3^-) and ammonia (NH_4^+) concentrations in the water and sand were determined according to the ISO standard methods, using a SEAL AutoAnalyzer 3HR (SEAL Analytical, WI, USA). Phosphate (PO_4^{3-}) phosphorous (P; Bray-2P extractant), sulphate (SO_4^{2-}), and sulphur (S) concentrations were determined with ICP analysis. The chemical oxygen demand (COD) was determined with a photometric kit (Spectroquant®; Merck-Millipore, Germany) according to the manufacturer's instructions. Total suspended solids (SS) was determined with vacuum filtration and oven drying. The pH of the water and sand slurries (1:1 w/v sand:deionised water) was measured with a Martini Mi150 pH meter (Milwaukee Electronics Kft., Szeged, Hungary). One millilitre of each water sample and 1 g of each of the three sand fractions were serially diluted and subsequently plated onto MacConckey (BioLab, Merck, Germany) and *Salmonella/Shigella* selective agar (Difco, Becton Dickinson and Company, NJ, USA). Faecal coliform and *Salmonella/Shigella* colonies were enumerated after overnight incubation at 44.5 °C and 37 °C respectively.

2.4 PLFA analysis and DNA extraction

Phospholipids were extracted from freeze-dried sand using the Bligh and Deyer method (Bligh and Deyer, 1959), and then transesterified. Fatty acid methyl esters (FAMEs) were analysed using GC-MS (Supplementary material). Total genomic DNA was isolated and

purified using a ZR Soil Microbe DNA MiniPrep™ kit (Zymo Research Corp., USA) according to the manufacturer's protocol. The extraction yield was evaluated by electrophoresis in 0.8 % (w/v) agarose gels stained with ethidium bromide, and the bands were subsequently compared to a 1000 bp DNA ladder (GeneRuler, Fermentas).

2.5 High throughput sequencing and sequence quality control

The V4-V5 region of the bacterial 16S rRNA gene, >400 base pairs (bp), was amplified by polymerase chain reaction (PCR) using a set of 23 uniquely barcoded forward primers and one reverse primer (Table S3.1, Supplementary materials). After size selection, the amplicons were templated and enriched using the Ion PGM™ Template OT2 400 Kit on the Ion OneTouch™ 2 System. The samples were loaded onto Ion 318 Chips for unidirectional multiplex sequencing using the personal genome machine (PGM™; Ion Torrent, Life Technologies). Raw sequencing reads were consecutively checked for different quality criteria (supplementary material) using the open source bioinformatics program MOTHUR v.1.34.4 (Schloss et al., 2009) installed on the University of Stellenbosch's Rhasatsha high performance computing network (Möller, 2014). Unique, non-chimeric sequences greater than 400 bp were aligned to the Silva reference database (Pruesse et al., 2007). The 16S rRNA data from individual samples were rarefied to equal sample size based on the sample with fewest sequences (444). Raw sequence data was submitted to the INSDC (EMBL-EBI/ENS, Genbank, DDBJ) with accession number DRA003988.

2.6 Statistical analysis

Commonly used indices of α-diversity, namely Shannon diversity and Chao1 richness (Begon et al., 1996; Chao and Shen, 2003; Hughes et al., 2001), which may be influenced by the addition of wastewater, were calculated using MOTHUR v.1.34.4 (Schloss et al.,

2009). All other calculations and statistical analyses were performed in the R v3.2 software environment (R Development Core Team, 2015). Analyses of variance (ANOVA) and pairwise t tests with Benjamini-Hochberg p value adjustment were performed on the sand chemical properties, α-diversity and richness. Differences in bacterial community composition were evaluated through permutational multivariate analysis of variance (PERMANOVA, 5000 permutations) and visualised using non-metric multidimensional scaling (NMDS, Vegan Community Ecology Package V2.3-0; Oksanen, 2015). Significant correlations of the physico-chemical variables with the NMDS ordinations were determined using least squares linear vector fitting, after the variables were subjected to z-score standardization. The significance of the fitted vectors was determined by 1000 permutations, and a Pr (>r) value < 0.05 was judged to be significant.

2.7 Correlation and network analysis

Spearman rank correlations between OTUs with more than 5 sequences were calculated to identify potential competition or cooperative interactions between OTUs in each sand fraction (Barberán et al., 2012; Cardinale et al., 2015). Strong correlations (R >= 0.7 or <= −0.7, and P <=0.01) were visualized through network analysis using Cytoscape 3.2.1, applying the Fruchterman-Reingold layout (Shannon et al., 2003). Non-random co-occurrence patterns were tested with the checkerboard score (C-score) under a null model preserving site frequencies (Stone and Roberts, 1990).

3 Results

3.1 Bacterial community relative activity and diversity

The activity of microbial communities in the sand fractions was confirmed when 12 PLFAs were identified (Figure S3.1) that are most likely indicators of bacterial activity and included saturated (14:0, 15:0, 16:0, 17:0, 18:0), unsaturated (16:1, 18:1, 18:2) and methyl

branched (13Me14:0, 10Me16:2, 15Me16:0, 10Me18:0) fatty acids (Andresen et al., 2014; Butler et al., 2003; Frostegård et al., 1993; Frostegård et al., 2011; Watzinger et al., 2008; Zhao et al., 2012). A total of 69 699 bacterial OTUs were obtained after “denoizing”, normalization and clustering at the 1 % cut-off level of 16S rDNA sequences (Table S3.2). No significant change in OTU α-diversity and richness was observed upon addition of the formulated influent onto the rhizofilter (Figure 3.1A and B). Also, the α-diversity in the three sand fractions remained similar over the three sampling months (Figure 3.1C). Concomitantly, OTU richness remained similar for all sand fractions analysed over the three sampling months. The exception was the planted bulk sand sampled in April, which showed a significantly higher OTU richness than most other monthly sand fraction samples (Figure 3.1D). In general, no significant difference could be observed between the three sand fractions with regard to either diversity or richness (Figure 3.1E and F).

OTU based β-diversity community analysis revealed that the microbial communities in the planted bulk and rhizosphere sand fractions were similar however, a significant difference ($p < 0.05$) was observed between the sand fractions of the planted and unplanted sides of the rhizofilter (Figure 3.2). The sand fractions and sampling months respectively accounted for 8 % and 6.15 % of the variability in the OTU dataset. Monthly variation was especially evident in the unplanted control, where three distinct clusters corresponding to the sampling months were observed (Figure 3.2). In contrast, the communities of the planted sand samples appeared to be more stable over the three months. Nevertheless, the microbial communities in the planted sand fractions sampled in April and May were more similar than those sampled in July (Figure 3.2). The time groups (0 to 168 h) explained 2.4% of the OTU variance.

3.2 Interaction between chemical factors and microbial communities

The formulated influent (pH 6.5 ± 0.5) contained nutrient levels and microbiological indicator concentrations typical of urban effluent (Table S3.3; Britz et al. 2013; Taebi & Droste 2004; Jamwal et al. 2008), while the sand showed no significant difference in pH between the control (5.3 ± 0.5), planted bulk (5.6 ± 0.4) and rhizosphere (5.9 ± 0.6) fractions. Fitted vectors of microbiological and chemical sand properties revealed that the OTU β-diversity correlated with faecal coliform, NO_3^-, S, P, and C concentrations, as well as the C:NH_4^+ ratio (Figure 3.2). Diversity and richness metrics also correlated with changes in OTU data. In addition, most of the PLFAs were significant in explaining changes in the β-diversity, indicating that differences in diversity can be related to differences in the activity of the system. Notably, all the methyl branched PLFAs pointed to the samples taken in July, possibly indicating higher activity of actinomycetes (Frostegård et al., 2011; Andresen et al., 2014) and Gram positive bacteria (saturated 16:0, 18:0 and mono-unsatured 16:1 PLFAs; Andresen et al., 2014; Butler et al., 2003; Frostegård et al., 2011) during this month. Samples taken in July had lower nutrient concentrations, since most of the chemical indicators pointed towards samples taken in April and May. It is also evident that the planted sand fractions had higher nutrient concentrations than the control. Although no significant difference could be observed between the sand fractions with regard to α-diversity and richness, the vector fitting analysis showed that these parameters tended to be higher in the planted sand fractions. The relative abundance of Gram negative (saturated 14:0 and 17:0 PLFAs; Andresen et al., 2014; Butler et al., 2003; Frostegård et al., 2011) PLFA markers were higher in April and May, compared to July.

3.3 Taxonomic composition

Eighteen bacterial phyla, including candidate divisions, were identified. Representatives of Actinobacteria were the most abundant in almost all of the samples (Figure 3.3). We

observed a 15 to 20 % increase in the relative abundance of the Actinomycetales in the rhizosphere and planted bulk sand fractions within 24 h after addition of the formulated influent (Figure S3.2). However, in the control sand fraction, the relative abundance of these bacteria appeared to decrease immediately after addition of the formulated influent, where after it returned to the original value within 168 h. This decrease in abundance of Actinobacteria could partially be ascribed to changes in the sand microbiota brought about by addition of microbial populations from the influent. Analyses of all sand fractions collected in July, one hour after addition of the influent, showed Pseudomonadales to be dominant (Figure 3.3). Within 24 h however, the Actinomycetales returned to its dominant status in the planted sand fractions, while it took longer for the relative abundance of these bacteria in the control sand to return to a comparable value observed during the other two months.

Bacterial families with a relative abundance greater than 1 % are shown in Figure 3.4. Phylogenetically, a clear distinction was observed between the unplanted and planted sand microbial communities. Nevertheless, families consistently present in all three sand fractions included Acetobacteriaceae, Acidobacteriaceae, Geodermatophilaceae, Intrasporangiaceae, Microbacteriaceae, Mycobacteriaceae, Nocardiaceae, Nocardioidaceae, and potentially novel species within the phylum Proteobacteria. Differences in the relative abundance of these bacteria could be observed between the planted and unplanted sand fractions. Notably, the Mycobacteriaceae and Nitrospiraceae, were more abundant in the planted sand than in the control sand, where the Geodermatophilaceae, Methylobacteriaceae and Sphingomonadaceae were more abundant.

Within these abundant families, consistently occurring high abundance OTUs were

identified by means of the indicator species algorithm in MOTHUR (Dufrêne and Legendre, 1997; Schloss et al., 2009). Representative sequences of these OTUs were aligned to the NCBI nucleotide database using the Basic Local Alignment Search Tool (BLAST). Sequences from the control showed the greatest homology with sequences of *Blastococcus* sp., *Methylobacterium radiotolerans*, *Modestobacter multiseptatus* and *Sphingomonas* sp. (Figure S3.3). In the planted sand fractions the highly abundant OTUs differed from that of the control, with OTU sequences aligning with those of *Mycobacterium brisbanense* (Figure S3.3). This was the only indicator species in the planted bulk sand samples. This species forms part of the *Mycobacterium fortuitum* third biovariant complex, which includes rapidly growing ubiquitous environmental organisms that normally inhabit soil, dust and water (Schinsky et al., 2004). Other indicator species of the rhizosphere sand were *Methylobacterium* spp. and the facultative methylotroph *Hyphomicrobium denitrificans* (Urakami et al., 1995).

3.4 Bacterial community interactions

Pollutant removal from wastewater often depends on interactions between metabolically distinct groups of bacteria. To explore potential syntrophy, competition or co-occurrence of bacteria, we used the OTUs to create non-random species association networks from strong ($r \geq 0.7$ or $r \leq -0.7$) and significant ($p < 0.01$) Spearman correlations for each sand fraction (Figure 3.5). We found that the number of nodes (n) and edges (e) in the control (n=66, e=136) were less than half of that in the bulk (n=151, e=328) and rhizosphere (n=160, e=387) sand fractions. Not only did the microbial communities differ between the unplanted control and planted sides, but also the interactions between these communities and nutrients present in the rhizofilter. Nevertheless, on both sides the most abundant OTUs correlated with the environmental variables. Spearman correlations between OTUs showed that co-occurrence prevailed over co-exclusion.

In the control sand fraction, sequences classified to the α-proteobacterial families Acetobacteriaceae, Methylobacteriaceae and Sphingomonodaceae co-occurred (Figure 3.5A). These results are consistent with those of others demonstrating that taxonomically related OTUs often co-occur (Cardinale et al., 2015; Faust and Raes, 2012). OTUs of the actinobacterial family Geodermatophilaceae showed negative correlations with methyl side chain PLFAs, as did OTUs of the Sphingomonadaceae. Representatives of Pseudomonadaceae showed low abundance in most of the samples, except during the third sampling month (Figures 3.3 and 3.4), and had negative correlations with the Sphingomonodaceae. OTUs of the Sphingomonodaceae correlated positively with most of the chemical properties, as well as with the saturated 14 carbon length (14:0) PLFA, a biomarker for Gram negative bacteria. The most abundant genus of the Sphingomonodaceae in the control sand fraction was *Sphingomonas*, which contains many species capable of degrading recalcitrant compounds (Leung et al., 1999; White et al., 1996).

In the planted sand fractions the Mycobacteriaceae had predominantly positive interactions with the Acetobacteraceae and Xanthomonadaceae (Figure 3.5B and 3.5C). The most abundant genus of the Xanthomonadaceae in the planted sand fractions was Rhodanobacter, which contains species thought to be important in soil denitrification processes (Kostka et al., 2012). Representatives of this genus are known to be dominant in bacterial communities occurring within some acidic and nitrate-rich environments. Another tentatively identified denitrifier was *Hyphomicrobium denitrificans* (Figure S3.3), which showed positive correlation with some of the Mycobacteriaceae OTUs.

Nitrifying bacteria, belonging to Nitrospiraceae, also co-occurred with some of the Mycobacterium OTUs. The Nitrospiraceae occurred more consistently in the planted sand fractions (Figure 3.4) and at a higher relative abundance than in the control sand fractions. Furthermore, the OTUs of the Nitrospiraceae in the rhizosphere sand fractions correlated with NO_3^- concentrations. In contrast to the control sand fraction, only a few correlations were observed between OTUs and chemical variables in the planted sand fractions. These were mostly negative correlations between OTUs of Mycobacterium and the C:N:P, $C:NO_3^-$ and C:N ratios.

3.5 Short term changes in microbial activity and community composition

In all three sand fractions Gram positive bacterial PLFA markers were the most abundant (Figure 3.6). The relative abundance of lipid biomarkers specific for Actinobacteria and Gram negative bacteria was much less. However, a significant portion of the Gram positive bacteria represented by the PLFAs were Actinobacteria. This was evident in the OTU based analysis (Figure 3.6) where OTUs of the Actinobacteria were much more abundant than OTUs of Gram negative bacteria in both the rhizosphere sand fractions. OTUs of Gram positive bacteria other than the Actinobacteria (e.g. Firmicutes) were rare. In the control sand fraction there appeared to be no difference between Actinobacteria and Gram negative OTU relative abundances. This was despite a significant difference in the relative abundance of PLFAs representing these two bacterial groups.

Changes in the PLFAs over time generally followed the same pattern as that of the OTUs, although there appeared to be a lag in the dynamics of the OTUs (Figure 3.6). For instance, an increase in relative abundance of Gram positive PLFAs was observed immediately after wastewater was added, whereas the increase in actinobacterial OTUs was only observed 24 h later. Furthermore, the relative abundance of Gram positive and

Gram negative PLFAs converged within 168 h, especially in the planted sand fractions. A similar but less pronounced pattern was observed in the OTU data. The process appeared to occur at a slower rate, with convergence of the curves only evident before wastewater was added. The increase in the Gram positive PLFA relative abundance could be due to high levels of Actinobacteria in the formulated influent.

4 Discussion

This is the first study to combine HTS and PLFA analyses to describe the temporal dynamics of microbial communities and their interactions within a phytoremediation system that treats a formulated influent representing severely polluted urban runoff. The dominance of Actinobacteria is in contrast to other studies that reported Proteobacteria to be the most abundant bacteria in many different environments, such as the rhizosphere, agricultural soil, activated sludge, and constructed wetlands (Hu et al., 2012; Ju and Zhang, 2014; Ligi et al., 2013; Mendes et al., 2013). Physical conditions that might have selected for Actinobacteria include the intermittent addition of nutrient rich water, which was shown to increase actinobacterial populations in a constructed riverine complex (Ligi et al., 2013). Also, the dominance of a particular group of organisms is believed to be a characteristic of high nutrient environments (Paul, 2000). Since easily metabolizable organic compounds are rapidly removed from municipal wastewaters (Van Loosdrecht et al., 2008), the organic carbon in the formulated influent was probably recalcitrant. Therefore, readily available organic carbon may be limited, especially in the control sand fraction, creating further selective pressure for Actinobacteria, known for their ability to degrade recalcitrant compounds (Ghai et al., 2014).

Unlike the control, the planted fractions may have continually received nutrient inputs through root exudates, which have a pronounced effect on treatment processes (Arthur et

al., 2005; Liu et al., 2012; Sleytr et al., 2007). The similarity between the planted sand fractions (Figure 3.2) indicated that the effect of the root exudates may have reached beyond the narrow zone directly around the root, or a dense root network might have formed in the sand medium. This continuous presence of nutrients in the planted sand fraction may therefore have increased the stability of the microbial communities. In contrast, the microbial communities in the control sand fraction appeared to be influenced by variations in the nutrient composition of the sand. This was evidenced by the distinct NMDS clusters corresponding to the three sampling months between which nutrient concentrations differed (Figure 3.2). Also, there appeared to be a stronger correlation between OTUs and chemical variables in the control than in the planted sand fractions (Figure 3.5).

A stable microbial community is generally considered to be a critical factor for maintaining ecosystem stability, nutrient cycling efficiencies and long term sustainability (Ramond et al., 2012). An example thereof in this study is the consistently higher abundance of nitrifying bacteria in the planted fractions compared to the control (Figure 3.4). However, this did not create significantly higher NO_3^- levels in the planted sand. A possible explanation may be coupled nitrification-denitrification processes which rapidly removed the produced NO_3^-.

The significant difference in β-diversity between the planted and unplanted control sides could largely be ascribed to different actinobacterial populations. Microbial communities associated with plant roots are subjected to the selective pressures created by the plant, as well as the presence of other microbial species (Cardinale et al., 2015; Danhorn and Fuqua, 2007). In this study we found that the wetland plants in the rhizofilter created selective conditions for the proliferation of non-tuberculosis Mycobacteriaceae (NTM;

Figures 3.4 and 3.5). Although NTM are ubiquitous in soil environments (Kator and Rhodes, 2003) they could also have originated in the formulated influent. Families of Actinobacteria found in the rhizofilter, including the Mycobacteriaceae and Nocardiaceae, commonly occur in the aerobic treatment systems of municipal wastewater (Ju and Zhang, 2014; Tsang et al., 2008). These bacteria have been implicated in acute infections in immunocompromised humans and those suffering from obstructive pulmonary disease (Kator and Rhodes, 2003). However, transmission of NTM infection between people is not common, and surface waters have been implicated in the spread of NTM in the environment (Pavlik and Falkinham, 2010). This study therefore adds to the mounting body of evidence showing a link between polluted, high nutrient aquatic environments and the presence of NTM (Makovcova et al., 2014; Pavlik and Falkinham, 2010; Pickup et al., 2006).

Apart from the Mycobacteriaceae, bacterial genera containing potentially pathogenic strains namely *Burkholderia*, *Herbaspirillum*, *Pseudomonas*, *Staphylococcus* and *Stenotrophomonas* (Berg et al., 2005), were also found in the three sand fractions. This supports the findings of others that certain environmental niches including the rhizosphere, sands, and sediments, harbour bacterial strains capable of bivalent interactions with both plants and humans (Cardinale et al., 2015; Mansilha et al., 2010; Sabino et al., 2011; Yamahara et al., 2007). However, relatively little is known about their virulence relative to their clinical counterparts (Mendes et al., 2013).

The high concentrations of culturable faecal indicator bacteria, which exceeded levels prescribed in guidelines for natural environments (Figure S3.4), did not correlate with OTU data on the Enterobacteriaceae. Sequences representing this commonly used group of indicator bacteria were mostly below the detectable limit of the HTS technique. It is

possible that the plate count techniques were not specific to their target indicator organisms, and that false positive colonies caused an overestimation of these groups of bacteria. However, we found genetic evidence of other gut commensal bacterial families, including the Campylobacteraceae, Moraxellaceae, Porphyromonadaceae and Prevotellaceae (Kator and Rhodes, 2003; Viau et al., 2011). The relative abundance of these bacteria was very low (<1 %), which is not surprising, since most of these environmentally allochthonous bacteria are adapted to low oxygen concentrations (Wexler, 2007).

In conclusion, the combined power of PLFA and HTS to assess structural shifts and activity in soil microbiota provided novel information regarding the effect of severely polluted water on microbial communities in a mimicked riparian environment. Variations in OTU abundance data could be related to changes in activity evidenced by PLFA analysis. Rhizofiltration could be useful in the removal of chemical pollutants due to the presence of beneficial functional groups of bacteria. Also human pathogens and commensialistic bacteria do not survive in the system. However there is some concern that phytoremediation systems can create selective conditions for potentially harmful organisms. Admittedly, many factors could affect the survival of potential pathogens in these systems. For that reason more studies should be done on the dissemination of potential pathogens outside the scope of enteric bacteria.

5 References

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Figures

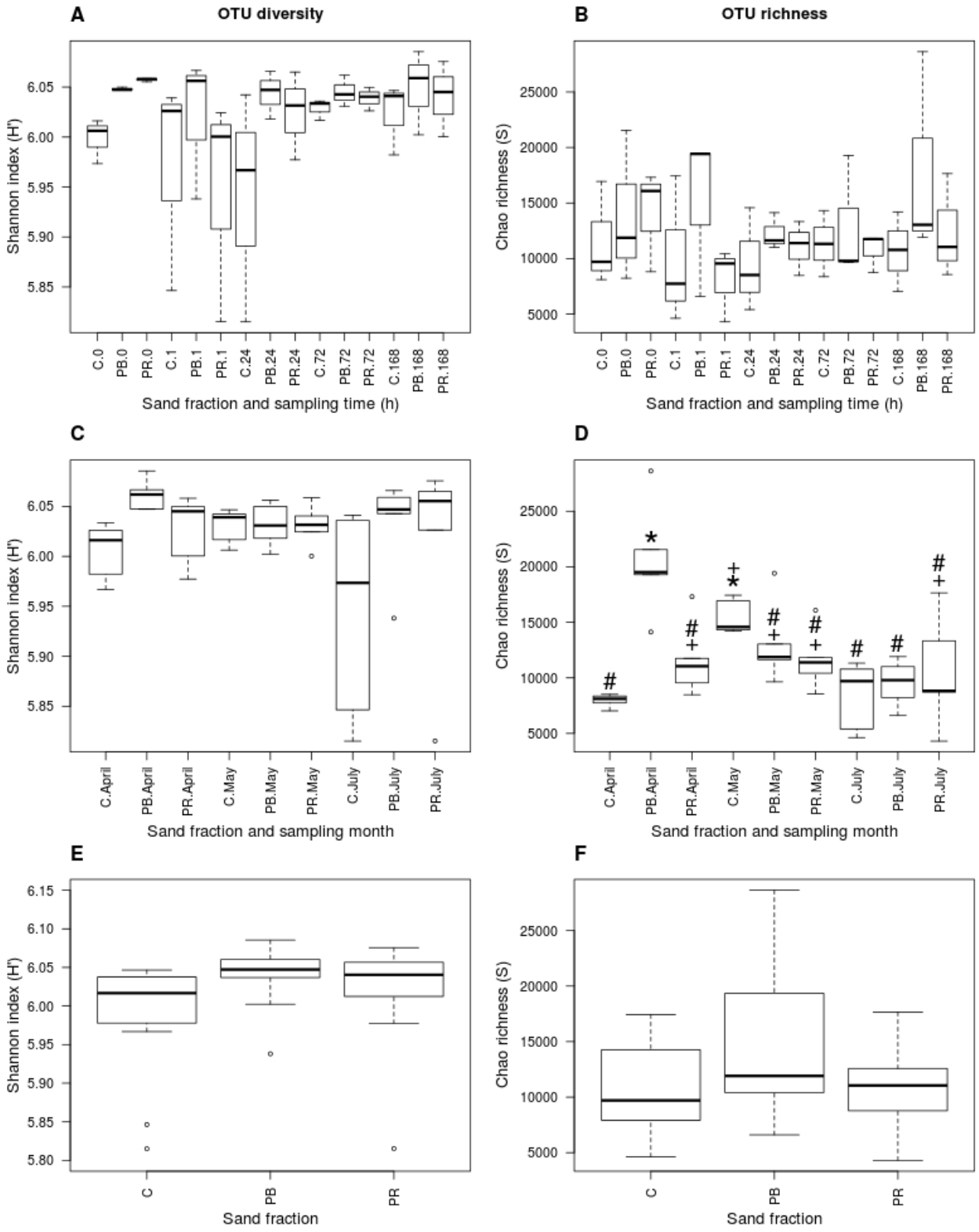

Figure 3.1: Boxplots of OTU diversity and richness of the three sand fractions, calculated using the Shannon diversity index and Chao richness estimate respectively. C=Control sand, PB= planted bulk sand, PR=rhizosphere sand. Symbols (D) indicate groups that were not significantly different (adjusted pairwise t test $p > 0.05$).

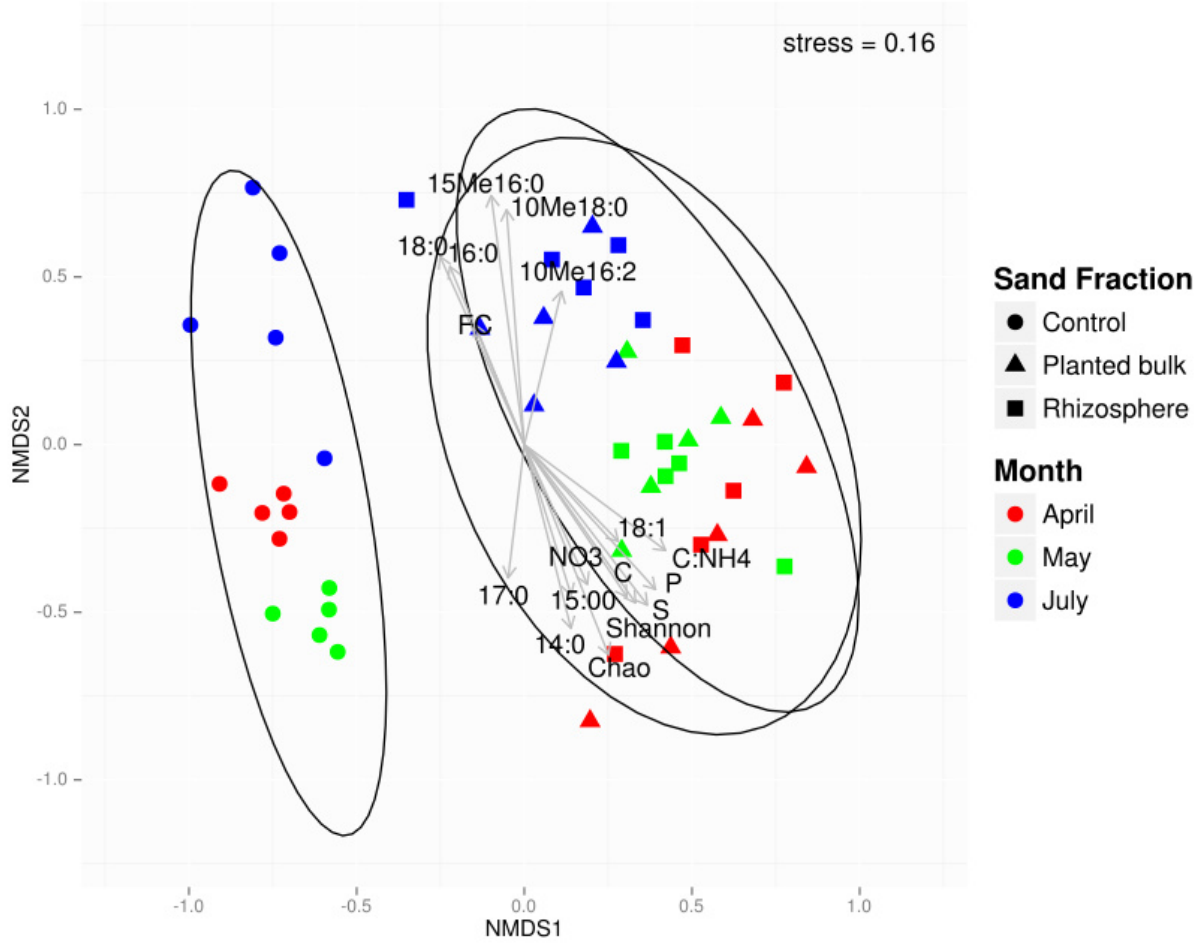

Figure 3.2: Nonmetric multi-dimensional scaling bi-plots showing the relative differences in OTU community composition. The Bray-Curtis distance metric was used to quantify the similarity between the observed patterns. Arrows (fitted vectors) indicate significance and direction of sand property correlations with the ordinations. Total carbon (C), carbon/ammonium ratio ($C:NH_4$), Chao richness, faecal coliforms (FC), nitrate (NO_3^-), phosphorus (P), Shannon diversity, and sulphur (S). PLFA -chain length:number of double bonds; Me = methyl side chains. Arrow length is proportional to the strength of the correlation (r). The significance of fitted vectors was determined using permutation tests (n=1000) at the $p<0.05$ significance level. Ellipses indicate a 95% confidence interval for replicates. The addition of a third dimension did not lead to a significant increase in the explanatory power of the ordinations.

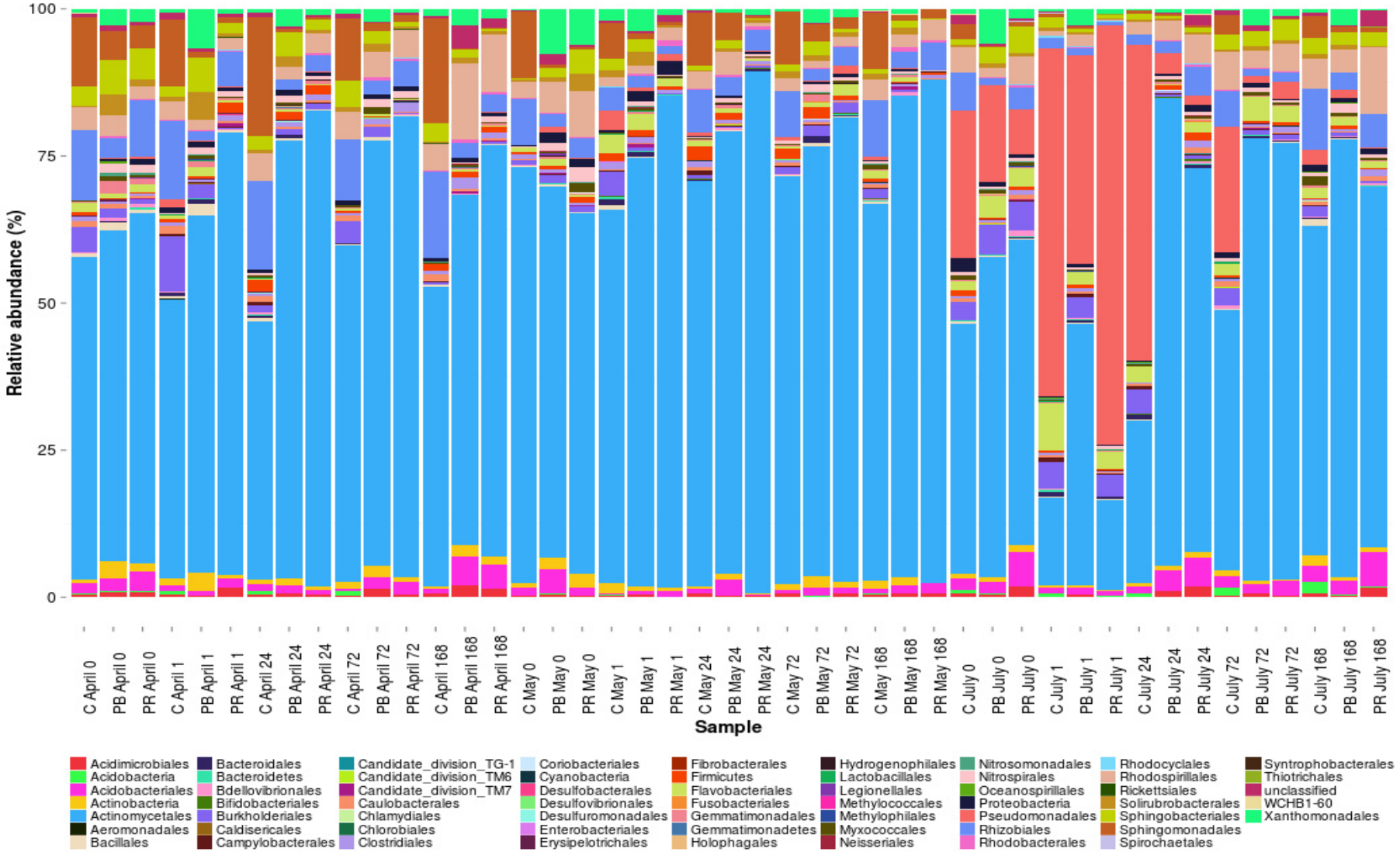

Figure 3.3: Bacterial community structures of the 45 sand samples. The percent abundance of each bacterial order in a sample is shown. Sequences were classified using Mothur and the Silva Gold 16S rRNA gene database with a confidence threshold of 80%. Sample codes include the month, time (before 0 and 1-168 h after wastewater was added) and sand fraction (C= unplanted control sand, PB= planted bulk sand, PR= rhizosphere sand).

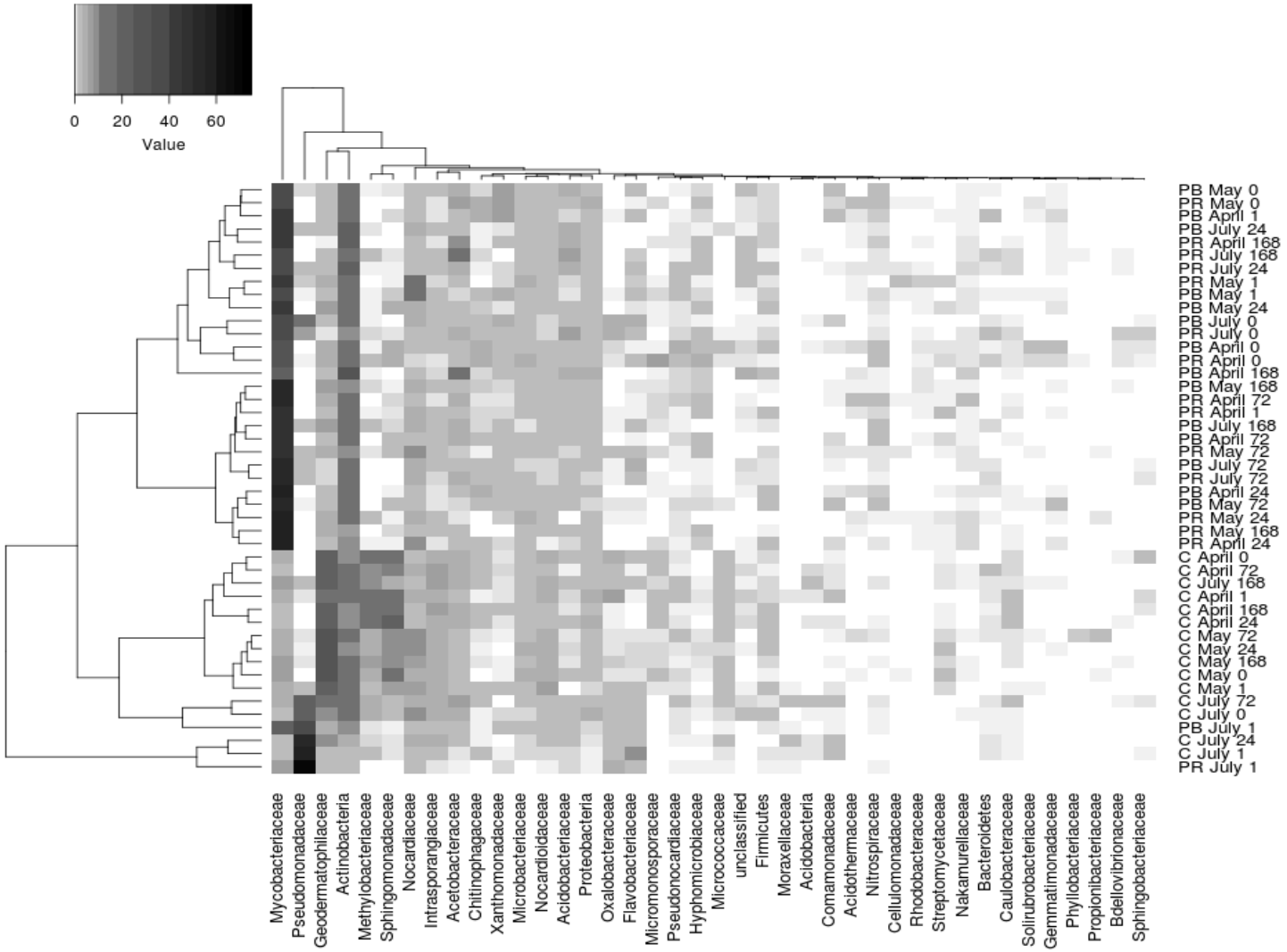

Figure 3.4: Heat map of relative abundances of the most abundant (>1%) OTUs in each of the 45 samples and hierarchical cluster analysis presented as a double dendrogram. OTUs were classified to the family level where possible and clustered according to abundance, not phylogenetic similarity (x-axis). Sample code indicates sampling month, and time of sampling, i.e. before (0h), and after (1-168 h) settled sewage was added to the system. Sand fractions: C= unplanted control sand, PB=planted bulk sand, PR=rhizosphere sand.

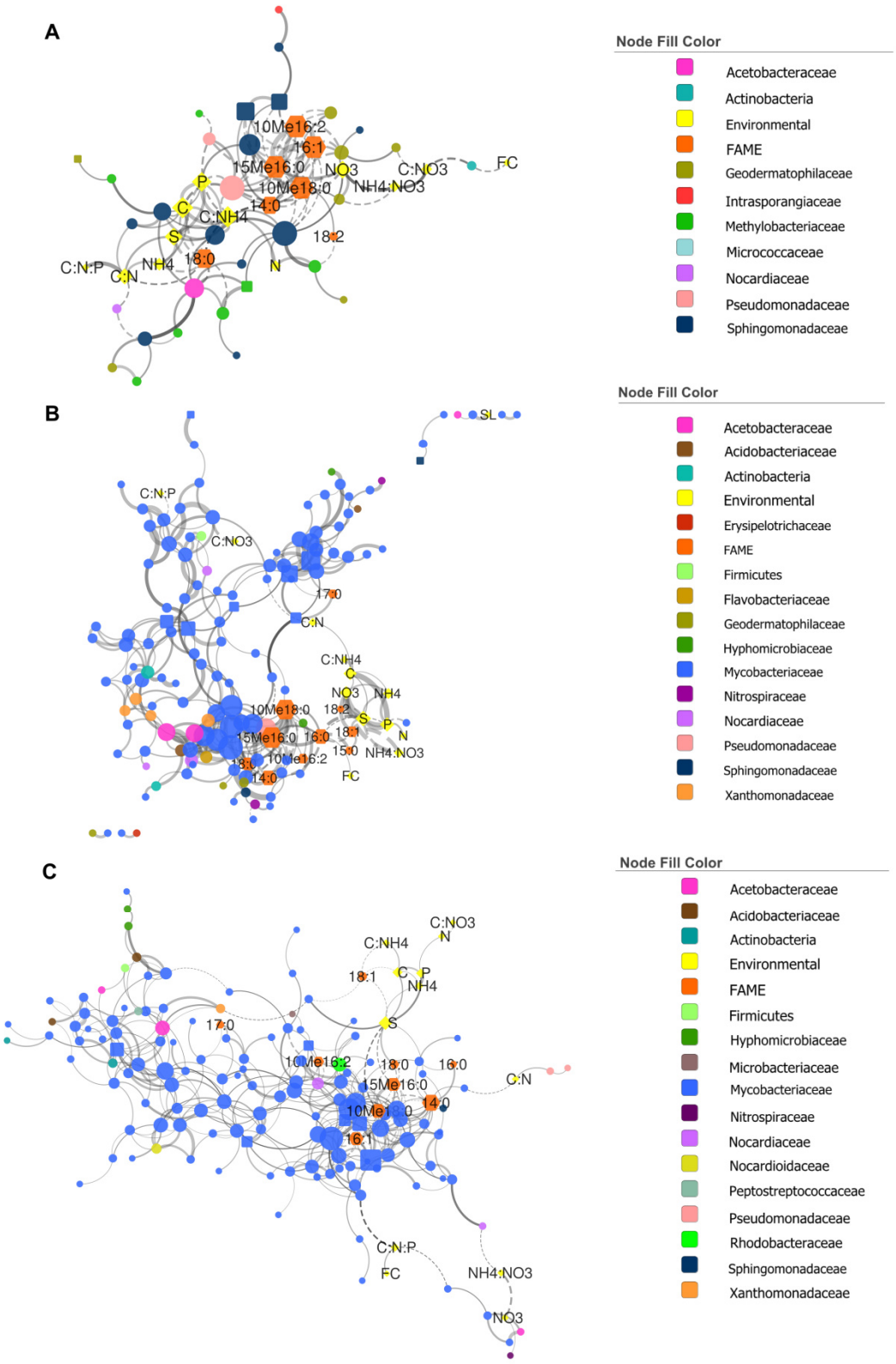

Figure 3.5: Non-random OTU co-occurrence networks and correlations with environmental variables. Spearman correlations with an r value >= 0.7 (solid edges) and <= -0.7 (dashed edges) at a confidence level of 95% were used to make the networks. Total carbon (C), carbon/ammonium ratio ($C:NH_4$), Chao richness, faecal coliforms (FC), nitrate (NO_3^-), phosphorus (P), Shannon diversity, and sulphur (S). FAME-chain length:number of double bonds; Me-methyl side chains. A=unplanted control sand, B= planted bulk sand, C=rhizosphere sand. Squares and circles indicate persistent (occurred in >80 % of samples), and transient OTUs (<80 %) respectively.

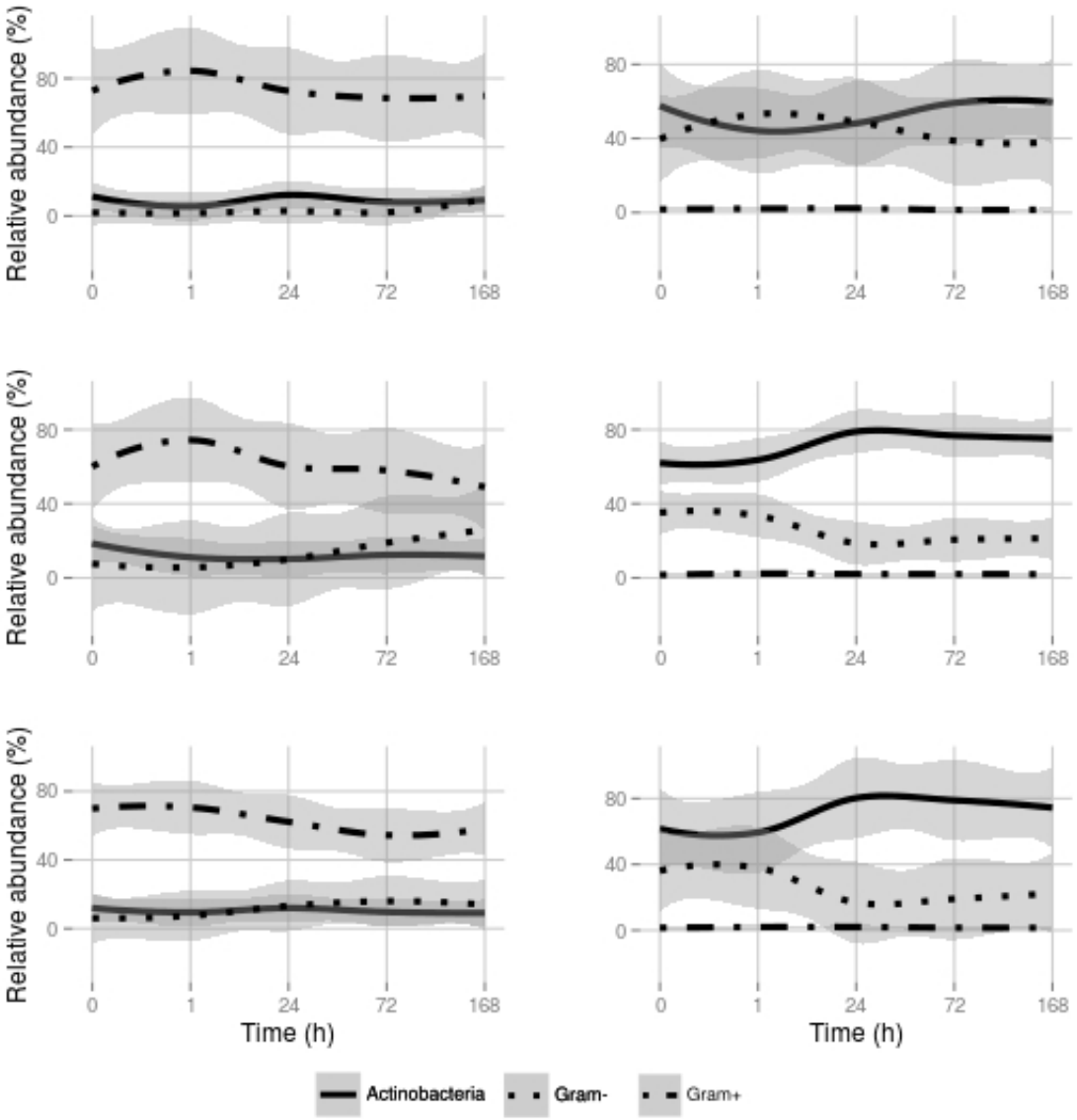

Figure 3.6: Comparison of FAME and OTU analysis of Gram negative, Gram positive and actinobacterial (which forms part of the Gram positive FAMEs) populations between the three sand fractions over a week. A=unplanted control sand, B= planted bulk sand, C=rhizosphere sand. Lines are LOESS smoothed curves, and the shaded areas indicate a 95% confidence level.

Chapter 4

Occurrence of opportunistic pathogenic yeasts in wastewater and their removal by rhizofiltration

1 Introduction

Considering the results of Chapter 3 it is clear that microbiological contaminants other than the faecal indicator bacteria could potentially be hazardous to people living near polluted rivers and streams. This also impacts on the availability of consumable fresh water which is decreasing globally due to exponential population growth and deteriorating water treatment systems (Gleick, 1998; Schwarzenbach et al., 2010). Additionally, water sources are threatened by both point and non-point source pollution, which may impact community health (Kim and Hur, 2010; Surbeck et al., 2010; Taebi and Droste, 2004). Water pollution in developing countries, including South Africa, is particularly problematic due to large semi-urbanized settlements which lack sufficient water supply and sanitation infrastructure (Govender et al., 2011; Karn and Harada, 2001; Oberholster et al., 2008; Qadir et al., 2010). Moreover, people living in these areas often use natural water courses for ablutions, irrigation and a source of potable water. Disturbingly, studies conducted on the water quality of South African rivers over the past decades, revealed an increase in pollution levels (Braune and Rogers, 1987; Britz et al., 2013; Greenfield et al., 2010; van Heerden et al., 2005). A contributing factor to such increased pollution levels in rivers is urban runoff, one of the most common forms of non-point source pollution. Urban runoff impacts water quality through the addition of sediment, toxic chemicals, elevated levels of nutrients as well as microbial pathogens (Jang et al., 2005; Walsh, 2000).

The main disease risk associated with drinking water in developing and transition countries is attributed to well-known pathogenic viruses, bacteria and protozoa, which spread via the fecal oral route (Schwarzenbach et al., 2010). Yeast populations, however, also constitute a significant component of aquatic microbial communities (Kwasniewska, 1988; Medeiros et al., 2008; Wurzbacher et al., 2010). The possible health risks accompanying the majority of waterborne yeasts have not been explored until now. A few species, primarily

within the anamorphic genus *Candida,* are able to grow at 37 °C and known to be important opportunistic pathogens (Brinkman et al., 2003; Cooper, 2010). When compared to clean water, polluted water contains higher concentrations of opportunistic pathogenic yeasts such as *Candida albicans*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilosis* (Medeiros et al., 2008; Stone et al., 2012; Valdes-Collazo et al., 1987). It has been suggested that the presence of these organisms in polluted rivers could be an indication of fecal contamination (Medeiros et al., 2008).

Candida albicans has long been considered the most dominant opportunistic pathogen in the genus *Candida* (De Hoog, 2000). However, recent studies revealed that other species, such as *Candida tropicalis*, *Candida dubliniensis* and *Candida glabrata,* have emerged from clinical cases (De Hoog, 2000; Guinea, 2014; Kothavade et al., 2010). It was suggested that the occurrence of these human-associated yeasts in aquatic environments pose an increased risk to public health (Blignaut et al., 2002; Medeiros et al., 2008; Silva et al., 2012). Some studies revealed that potentially fatal infections could occur when immunocompromised individuals consume water contaminated with the abovementioned *Candida* species (Hazen, 1995; Kothavade et al., 2010; Niewerth and Korting, 2002). This is highly probable in countries such as South Africa with high numbers of human immunodeficiency virus (HIV) infected individuals (Abrantes et al., 2013; Blignaut et al., 2002; Evans et al., 2012; Silva et al., 2012). Moreover, pathogenic *Candida* strains have been shown to carry resistance towards antifungal drugs (Silva et al., 2012).

Therefore, in water management, it is essential to intercept and treat urban runoff before it enters natural water bodies. Historically, urban point source pollution was the primary target in wastewater management through the establishment of wastewater treatment works (WWTW). In contrast, no constructive planning or any effective measures have

been taken to manage urban non-point sources of pollution (Taebi and Droste, 2004). However, in recent years this aspect has received much needed attention and several strategies have now been proposed.

Phytoremediation of various effluent types using constructed wetland technologies have proved to be effective at reducing the concentration of nutrients, fecal indicator organisms and pathogens usually found in wastewater (Calheiros et al., 2007; Kivaisi, 2001; Kurzbaum et al., 2012; Zhang et al., 2010). These water treatment technologies offer a suitable combination of physical, chemical and biological factors for the removal of pathogenic organisms. However, the removal of potentially pathogenic yeasts (PPY) has not been investigated.

In this study, a pilot scale low impact development best management practice (LID-BMP) was used to investigate the removal of PPY from urban runoff. This LID-BMP, called a rhizofilter, combines aspects of constructed wetlands, sand and trickling filter technologies (Arthur et al., 2005; Dushenkov et al., 1995; Mthembu et al., 2013). The main mechanism of pollutant removal is hypothesized to occur through the rhizomes of wetland plants selecting for functional microbial communities that drive nutrient mineralization processes such as nitrification (Chapter 3). The aim of this study was thus to evaluate the performance of the rhizofiltration system in terms of PPY removal. We also investigated the effect of physicochemical parameters on the PPY populations in wastewater before and after treatment. Furthermore, using next generation sequencing (NGS), we aimed to determine the relative abundance of PPY species in the mycobiome of the rhizofilter.

2 Materials and methods

2.1 Experimental setup

The pilot scale rhizofilter described in Chapter 1 was used for this study (Figure 1.1). Monthly experiments in which the system was operated in batch flow mode, simulating pulse discharge of urban effluent, were conducted from November 2011 until October 2013. Settled sewage, from the Stellenbosch WWTW, was pumped into a 10 000 L settling tank. Further settling for four days resulted in a formulated influent with microbiological and chemical characteristics similar to that of urban effluent reported in literature (Britz et al., 2013; Jamwal and Mittal, 2010; Taebi and Droste, 2004; Taylor et al., 2005).

Excessive solids which might result in clogging of the rhizofilter was removed by flushing 2 000 L of the formulated influent using the system's bypass valve. Subsequently, 2 500 L of the formulated influent was released onto the rhizofilter at a rate of ca. 7 $L.s^{-1}$ and distributed equally over both the planted and unplanted sides via two sets of overhead valves. The formulated influent percolated through the system for 45 minutes and the effluent was collected from two outlets on both sides of the rhizofilter in 500 ml sterilized glass bottles. The temperature of the formulated influent, planted effluent and unplanted effluent samples was measured directly after sampling using a mercury thermometer. This experimental procedure was performed in triplicate and samples were processed immediately upon arrival at the laboratory.

2.2 Yeast isolation and enumeration

During performance testing of the system at each sampling event, we analyzed the culturable PPY community of the formulated influent. Water samples (500 ml) were aseptically collected from the 10 000 L settling tank. Subsequently PPY were isolated and enumerated from this formulated influent, as well as from the abovementioned samples

collected from the planted and unplanted sides of the rhizofilter. Yeast isolation was accomplished by filtering 100 ml of a 10^2 dilution of the samples through 0.45 µm pore cellulose nitrate filter disks (Sartorius Stedim Biolab Products Augbane, France) using sterile polycarbonate filter systems (Sartorius Stedim Biolab Products Augbane, France). The filter disks were then transferred directly onto Sabouraud Glucose Agar (SGA; Atlas, 1993) supplemented with chloramphenicol (30 µg.ml^{-1}), tetracycline (10 µg.ml^{-1}) and kanamycin (50 µg.ml^{-1}), and incubated at 37 °C for 48 hours until yeast colonies were clearly visible (Chowdhary et al., 2011). After incubation, the total yeast colony forming units (CFUs) were counted. A maximum of ten colonies were randomly selected from the filter disks of each replicate sample. These colonies were purified by successive culturing at 37 °C on SGA and identified as described below.

2.3 Yeast identification

To rapidly identify PPY belonging to the genus *Candida*, pure cultures of the isolated yeasts from each water sample were spotted onto Molybdate Agar plates (Atlas, 1993; Bump and Kunz, 1968; Holland and Kunz, 1960) and incubated for four days at 37 °C. A photographic identification guide (Figure S4.1), was used to do preliminary identifications of the isolates based on colony colour and morphology. The accuracy of the photographic identification guide was confirmed by identifying random representatives of each morphological group using DNA sequence analyses of the D1/D2 region of the variable domains of the large 28S rDNA subunit (Supplementary material).

2.4 Physico-chemical analyses

The following physico-chemical parameters were measured to determine the effect of pollution on PPY populations in the water samples. Nitrate (NO_3^-) and ammonia (NH_4^+) concentrations in the water were determined according to the ISO standard methods using

a SEAL AutoAnalyzer 3 HR (SEAL Analytical, WI, USA). Chloride (Cl^-), phosphate (PO_4^{3-}), and sulphate (SO_4^{2-}), concentrations were determined using ICP analysis. The chemical oxygen demand (COD) was determined with a photometric kit (Spectroquant® ; Merck-Millipore, Germany) according to the manufacturer's instructions. Total suspended solids (SS) was determined with vacuum filtration and oven drying. The pH of the water was measured with a Martini Mi150 pH meter (Milwaukee Electronics Kft., Szeged, Hungary). To determine if PPY correlated with bacterial indicators of water pollution, 1 ml of each of the water samples were serially diluted and subsequently plated onto MacConckey (BioLab, Merck, Germany) and *Salmonella/Shigella* selective agar (Difco, Becton Dickinson and Company, NJ, USA). Faecal coliforms and *Salmonella/Shigella* colonies were enumerated after overnight incubation at 44.5 °C and 37 °C respectively.

2.5 Filter medium sampling

To characterize the mycobiome of both the planted and unplanted sides of the rhizofilter and to determine possible effect of wastewater addition thereon we collected representative samples of the sand layer on 18 April 2014. A 10 cm-diameter soil corer was used to collect sand samples to a depth of 15 cm immediately before, one, and 168 h after the addition of wastewater. The soil corer was sterilized with ethanol between sites. Three replicate samples (±250 g), each consisting of three pooled sand cores, were collected along the length of the control side. Replicate sand samples were collected from the planted side by removing five mature *T. capensis* plants (the dominant species) along the length of the filter. The plant rhizomes were vigorously shaken in a plastic bag to collect the sand fraction (±300 g each). Samples were transported on ice to the laboratory. All replicate samples were then pooled and homogenized by sieving the samples (2.8 mm mesh) removing debris at the same time. In total there were six composite samples, three

from each side of the rhizofilter, one from each time interval. These samples were stored at -20 °C until DNA extraction.

2.6 Fungal metagenome sequencing and sequence quality control

Total genomic DNA was isolated and purified using a ZR Soil Microbe DNA MiniPrep™ kit (Zymo Research Corp., USA) according to the manufacturer's protocol. Extraction yield was evaluated by electrophoresis in 0.8 % (w/v) agarose gels stained with ethidium bromide and bands were compared to a 1000 bp DNA ladder (GeneRuler, Fermentas). The internal transcribed spacer (ITS) region of the fungal 18S rRNA gene, was amplified by polymerase chain reaction (PCR) using a set of 6 uniquely barcoded forward ITS1f primers and one reverse primer (Table S4.1, Supplementary materials). After size selection the amplicons were templated and enriched using the Ion PGM™ Template OT2 400 Kit on the Ion OneTouch™ 2 System. The samples were loaded onto an Ion 318 Chip for unidirectional multiplex sequencing using the personal genome machine (PGM™; Ion Torrent, Life Technologies). Raw sequencing reads were consecutively checked for different quality criteria (supplementary material) using the open source bioinformatics programs QIIME (Caporaso et al., 2010) and PIPITS, an automated pipeline for analysis of fungal ITS sequences (Gweon et al., 2015). Unique, non-chimeric sequences greater than 160 bp were aligned to the UNITE (Abarenkov et al., 2010) reference database and clustered into OTUs at a 97 % similarity. Taxonomic identification was performed at an 80 % similarity cut-off value. The 18S rRNA data from individual samples were rarefied to equal sample size based on the sample with fewest sequences (4855). Raw sequence data was submitted to the INSDC (EMBL-EBI/ENS, Genbank, DDBJ) with accession number DRA004161.

2.7 Statistical analysis

All the physico-chemical variables were found to have a non-normal distribution after applying the Kolmogorov-Smirnov test for normality. Kruskal-Wallis analysis of variance (KW-ANOVA) of non-parametric data was done to determine significant differences in physico-chemical and microbiological properties between the formulated influent, unplanted control effluent and planted effluent. The PPY diversity of the formulated influent and effluents of the planted and unplanted sides was calculated with Shannon's diversity index (H').

Differences in PPY community composition between the influent and effluents of the planted and unplanted sides were evaluated through permutational multivariate analysis of variance (PERMANOVA, 5000 permutations). Non-metric multidimensional scaling was used to visualize the difference in PPY communities (NMDS, Vegan Community Ecology Package V2.3-0; Oksanen 2015). Significant correlations of the physico-chemical variables with the NMDS ordinations were determined using least squares linear vector fitting, after the variables were subjected to z-score standardization. The significance of the fitted vectors was determined by 1000 permutations, and a Pr (>r) value < 0.05 was judged to be significant.

Spearman's rank correlation coefficients were calculated to determine the effect of the physico-chemical characteristics of the wastewater on PPY concentrations and removal. Additionally, the random forest machine learning method was used to model the effect the physico-chemical characteristics of the wastewater might have on the PPY yeast concentration and the relative abundance of the yeast species (Cutler et al., 2007; Díaz-Uriarte and Alvarez de Andrés, 2006; Liaw and Wiener, 2002; Strobl et al., 2009). Species

abundance was also included as variables in the random forest analyses to give an indication on the co-occurrence of the PPY.

Differences in metagenome fungal community composition between the planted and unplanted sand was determined using the generalized UniFrac (GuniFrac) algorithm. This algorithm created weighted (α 0.5), unweighted and variance adjusted weighted distance matrices. Subsequently, significance was tested with GPERMANOVA on the three calculated distance matrices and principal coordinate analysis (PCoA) was used to visualize the result.

All statistical analyses were performed in the R statistical software environment (R Development Core Team, 2015) using the packages vegan (Oksanen et al., 2015), GUniFrac (Chen, 2012) and randomForest (Liaw and Wiener, 2002). Figures 4.1, 4.2 and 4.6 were created with ggplot2 (Wickham, 2009). Figure 4.6 was created with the googleVis package (Gesmann and De Castillo, 2011).

3 Results

3.1 Yeast Removal

The mean concentration of PPY in all samples (Table 4.1) taken from the effluent of both the planted (2.7 ± 2.2 log yeast CFUs.100 ml^{-1}) and unplanted (2.4 ± 1.7 log yeast CFUs.100 ml^{-1}) sides of the filter was significantly ($p < 0001$) lower, compared to the influent (3.7 ± 1.5 log Yeast CFUs.100 ml^{-1}). The mean PPY removal efficiency of all the sample data from the unplanted side (61.5 ± 55.5 %) appeared to be higher than the removal efficiency (50.4 ± 62.8 %) by the planted side. Although the removal efficiency did

not differ significantly between the two sides, the concentration of PPY in the respective effluents did show a significant difference (Table 4.1).

Significant variations were observed in the yeast concentrations of the formulated influent, unplanted effluent and planted effluent over the sampling period, which lead to cyclic variation in the yeast removal efficiency of the rhizofiltration system (Figure 4.1A). The planted side removed a higher numbers of yeasts in December 2011, and again from April 2012 to August 2012 (Figure 4.1B) when compared to the unplanted side. During the 2013 sampling months we observed the opposite, with the unplanted side generally having higher levels of yeast removal than the planted side. The planted side achieved 100 % removal of PPY from July to August 2012, while the unplanted side showed the highest removal efficiency in March 2013 to July 2013 (80 to 100 %). The variation in the PPY concentrations and removal could have been influenced by a number of physico-chemical factors.

3.2 Factors affecting PPY concentration and removal

Using Spearman's rank order correlation coefficient, statistically significant correlations were found between the yeast concentration, yeast removal efficiency and the physico-chemical properties of the formulated influent, unplanted and planted effluents (Table 4.2). The yeast concentration in the unplanted side's effluent only correlated with the influent-SS concentration. Yeast removal by the unplanted side correlated positively with the influent PPY concentration ($r = 0.474$, $p < 0.05$) and Cl^- removal ($r = 0.614$, $p < 0.01$). Negative correlations were found between PPY removal by the unplanted side and SS-removal ($r = -0.586$, $p < 0.01$), as well as the influent SS concentration ($r = -0.560$, $p < 0.01$).

The yeast concentration in the effluent of the planted side correlated positively with the influent yeast ($r = 0.822$, $p < 0.01$), PO_4^{3-} ($r = 0.518$, $p< 0.05$) and NH_4^+ ($r = 0.467$, $p < 0.05$) concentrations, as well as with the effluent NH_4^+ concentrations ($r = 0.536$, $p < 0.05$; Table 4.2). In contrast to the unplanted side, PPY removal by the planted side correlated negatively with the PPY concentration in the formulated influent ($r = -0.466$, $p < 0.05$). Furthermore, PPY removal by the planted side correlated with different variables than PPY removal by the unplanted side (Table 4.2). This included negative correlations with the water temperature ($r = -0.651$, $p < 0.01$), effluent NO_3^- ($r = -0.601$, $p < 0.01$), influent NO_3^- ($r = -0.560$, $p < 0.05$) and effluent SO_4^{2-} ($r = -0.486$, $p < 0.05$) concentrations. Interestingly, the only correlation with temperature was PPY removal by the planted side ($r = -0.651$, $p < 0.01$), showing improved removal efficiencies at lower water temperatures.

The formulated influent PPY concentration correlated with most of the physico-chemical variables except for temperature and the influent NH_4^+ concentration (Table 4.2). Positive correlations were found with the influent PO_4^{3-} ($r = 0.668$, $p < 0.01$), influent NO_3^- ($r = 0.509$, $p < 0.05$), influent SO_4^{2-} ($r = 0.571$, $p < 0.05$) and influent COD ($r = 0.499$, $p < 0.05$) concentrations. A negative correlation was found with SS ($r = -0.496$, $p < 0.05$) concentration.

Random forest analysis was used to model the importance of the physico-chemical properties on PPY concentration (Figure 4.2). We found that the concentrations of PO_4^{3-}, COD, NH_4^+, NO_3^-, SS and SO_4^{2-} were important chemical predictors of PPY concentration in the formulated influent. The PPY concentration in the effluent of the planted side was affected by water temperature, SO_4^{2-}, PO_4^{3-}, NO_3^-, NH_4^+ and to a lesser extent the COD. In the effluent of the unplanted side the water temperature, SO_4^{2-}, PO_4^{3-}, *Salmonella* and NH_4^+ were important predictors of PPY concentration.

3.3 Potentially pathogenic yeast communities

Strains of five dominant PPY species namely *Candida glabrata, C. krusei, C. tropicalis, C. utilis* and *Saccharomyces cerevisiae* were detected in the formulated influent and effluent samples, (Table 4.3, Figure 4.3). Three *Candida lusitaniea* strains and one *Pichia rhodanensis* strain were also isolated but due to limited abundance data these species were excluded from further analyses.

The PPY concentration in the formulated influent was influenced by the number of *C. utilis, C. tropicalis* and *C. krusei* strains, evidenced by their importance in explaining the variability in PPY concentration calculated using random forest analyses (Figure 4.2). Concentrations of PPY in the effluents from the planted and unplanted side were affected by different *Candida* strains than those in the formulated influent. In the effluent of the planted side *C. glabrata, C. tropicalis,* and *C. utilis* were important determinants of PPY concentration. In the effluent from the unplanted control, *C. glabrata* was the most prominent in explaining PPY concentration variance.

Permutational multivariate analysis of variance and NMDS of the PPY communities showed that there was a significant ($p<0.001$) difference between the influent and the effluents from both the planted and unplanted sides (Figure 4.4). The composition of the PPY in the unplanted effluent was the most stable; since the samples grouped closer together than what was observed for the planted effluent and formulated influent communities (Figure 4.4). Shannon's diversity index (H) values, which were fitted as a vector on the NMDS ordination plot (Figure 4.4), pointed to greater PPY diversity in the formulated influent and planted effluent compared to the unplanted effluent. The contours of NMDS ordination plot also indicated that the yeast concentration in the unplanted effluent was mostly lower than in the formulated influent and the planted effluent. Other

significant vectors fitted on the NMDS ordination included the SS and NH_4^+ which pointed to higher concentrations in the formulated influent.

3.4 Factors affecting PPY species abundance

Figure 4.2 indicates variables that are important in predicting the abundance of the individual PPY species. The relative abundance of *C. tropicalis* in the formulated influent was affected by the PO_4^{3-}, COD and SO_4^{2-} concentrations. Also, this species appeared to co-occur with *C. utilis* and *S. cerevisiae*. In the effluent of the planted side *C. tropicalis* was affected by the PO_4^{3-} and SO_4^{2-} concentrations. *Candida glabrata* and *C. utilis* appeared to co-occur with *C. tropicalis*. Predictions on factors that could influence the relative abundance of *C. tropicalis* in the effluent of the unplanted side could not be calculated due to the low abundance and infrequent occurrence of this species.

In the formulated influent the relative abundance of *C. glabrata* was predicted by the water temperature, SS, pH, Cl^- and to a lesser extent NH_4^+ (Figure 4.2). The presence of this yeast in the effluent of the unplanted side was predicted by the water temperature, NH_4^+ and SO_4^{2-} and weakly by the relative abundance of *S. cerevisiae*. In the effluent of the planted side, the COD concentration and relative abundance of *C. tropicalis* were strong predictors of *C. glabrata*. The aforementioned yeasts were the most abundant in the effluent of the planted side (Table 4.3, Figure 4.3).

Phosphate, NH_4^+, SO_4^{2-} and Cl^- concentrations predicted the relative abundance of *C. kruseii* in the formulated influent (Figure 4.2). The occurrence of *C. kruseii* in the effluent of the unplanted side could not be predicted by the measured physico-chemical variables except for the COD concentrations which was a relatively weak predictor. This yeast did

not occur frequently enough in the planted effluent to make predictions on properties that may affect its relative abundance.

The relative abundance of *C. utilis* in the formulated influent was predicted by the NO_3^-, faecal coliforms and COD concentrations as well as by the relative abundance of *C. tropicalis* (Figure 4.2). In the unplanted side's effluent, water temperature and PO_4^{3-} were the best predictors. Factors that predicted the relative abundance of *C. utilis* in the effluent of the planted side were water temperature, SO_4^{2-}, PO_4^{3-}, NO_3^- and COD concentrations. *Candida kruseii* was a weak predictor of *C. utilis* in the effluent of the planted side.

3.5 Fungal community in filter media

High throughput sequencing of the fungal metagenome in the planted and unplanted sand fractions showed that there was no significant difference in the α-diversity, evenness or richness (Table 4.4). The OTU richness, however, appeared to be higher in the planted compared to the unplanted side. There was a significant difference (GPERMANOVA of GUniFrac α1 distance <0.05) in β-diversity between the planted and unplanted sand fractions (Figure 4.5). The sampling time contributed to the observed variance especially in the planted sand.

The taxonomic composition of the fungal communities showed that the PPY species isolated in this study were mostly below the detectable limit of the HTS technique. Only two OTUs of *Candida* occurred in the dataset and they were identified as a *Candida* sp. and *C. tropicalis*, with a mean relative abundance of 0.17 % and 0.1 % respectively. Both of these OTUs were only found on the planted side.

The abundant (> 1 % relative abundance) OTUs differed between the planted and unplanted sand fractions (Figure 4.6). A large percentage (ca 38 -68 %) of the OTUs in the unplanted sand was unidentified fungi. Other abundant OTUs in the unplanted sand represented the Ascomycota and Basidiomycota which could not be identified to a lower taxonomic rank. Some Tremellomycete OTUs only occurred in the unplanted sand and not in the planted sand, while; other OTUs of Tremmellomycetes such as *Trichosporon ovoides* was more abundant in the planted sand. This yeast was, however, only a small fraction of the dominant (36.7 – 57 %) basidiomycete population in the planted sand (Figure 4.6).

The abundant Ascomycota OTUs, which could be identified at a lower rank, all belonged to the Pezizomycotina and included the following fungal classes: Dothidiomycetes, Letiomycetes, Pezizomycetes and Sordariomycetes (Figure 4.6). Abundant OTUs of the Sordariomycetes that could be identified at a lower rank were Chaetomiaceae and Zopfiella.

4 Discussion

In this study we showed that a pilot scale rhizofiltration system could remove PPY from wastewater representing polluted urban runoff with a mean reduction of 0.5 to 1 log CFUs.100 ml^{-1} (Table 4.1). The concentration of the PPY in the influent and effluents correlated positively with chemical indicators of pollution (PO_4^{3-}, NO_3^-, NH_4^+, SO_4^{2-} and COD) for both planted and unplanted sides (Table 4.2). These chemicals were also found to be important predictors of the PPY concentrations. Additionally, the water temperature was a predictor of the PPY concentration in both effluents but not of these yeasts in the influent. However, there was a significant negative correlation between water temperature and yeast removal by the planted side of the rhizofilter. Higher temperatures may therefore

facilitate the growth of PPYs in the rhizofilter between performance testing events, which would reduce the removal efficiency.

Analysis of the yeast species, that constituted the PPY population in the wastewater samples, revealed that the influent yeast population differed from both the effluents of the unplanted and planted sides. We also found that the PPY concentrations in the influent and effluents were affected by different *Candida* species (Figure 4.2). Furthermore, the relative abundance of each PPY species found in this study was shown to be predicted by different combinations of the chemical pollutants. The PO_4^{3-} concentration, for example, only predicted the relative abundance of *C. krusei* and *C. tropicalis* in the influent. Strains of these species were isolated more frequently from the formulated influent compared to either of the effluents (Table 4.3, Figure 4.3). The aforementioned species were also important predictors of the influent PPY concentration. Phosphate is excreted through human and animal faeces, confirming the findings of other studies linking increased concentrations of this growth limiting macronutrient in polluted waters to microbial indicators of faecal pollution, including PPY (Carrillo et al., 1985; Hagler et al., 1986; Ortega et al., 2009; Stone et al., 2012).

Additionally, we found evidence that nitrification affected the PPY population. The NO_3^- concentration was an important predictor of the total PPY concentration in the influent and effluent of the planted side of the rhizofilter (Figure 4.2). In particular, the relative abundance of *C. utilis* in the planted effluent was predicted to be influenced by the NO_3^- concentration (Figure 4.2). The relative abundance of *C. utilis* appeared higher in the planted effluent than in the unplanted effluent and formulated influent (Figure 4.3). Concomitantly, the NO_3^- concentration in the planted side's effluent tended to be higher than in the effluent of the unplanted side and the formulated influent (Table 4.1). *Candida*

utilis is known to assimilate NO_3^- and it is tempting to speculate that the proliferation of this yeast in the rhizofilter may be enhanced when NO_3^- is present (Eddy and Hopkins, 1985; Hipkin et al., 1990). It must be noted however, that growth and survival of a particular yeast species within an ecosystem may not solely depend on the intrinsic abilities of the yeast, but is the cumulative result of a number of abiotic and biotic interactions within that particular ecosystem (Botha, 2006).

The significantly lower NH_4^+ concentrations in the system's effluents indicated that nitrification occurred between performance testing events, removing NH_4^+ from both the unplanted and planted media (Table 4.1). Ammonium concentrations correlated with changes in the PPY communities (Figure 4.4) as well as the PPY concentrations in the planted effluent. Moreover, NH_4^+ was found to be an important predictor of the PPY concentrations in the formulated influent and the effluent from both the planted and unplanted sides of the rhizofilter (Figure 4.2). Ammonium was also an important predictor for *C. glabrata* and *C. kruseii* in the influent as well as *C. glabrata* in the effluent of the planted side (Figure 4.2). These observations may have been as a result of indirect interactions between the different yeast populations and NH_4^+ levels. However, it may also indicate metabolic differences between the yeast species regarding NH_4^+ assimilation. It is has been known for some time that *Candida* species may differ with regard to the enzymes involved with NH_4^+ assimilation (Holmes et al., 1989). For example, the glutamate synthase (GOGAT) enzyme was shown by Holmes and co-workers (1989) to have a higher activity in *C. albicans* compared to other strains of species in the same genus.

The PPY species isolated in this study are known to occur in municipal wastewater (Biedunkiewicz and Baranowska, 2011; Biedunkiewicz and Ozimek, 2009). However,

during this study we did not isolate any strains representing the notorious fungal pathogen *C. albicans.* Recent studies on the occurrence of *C. albicans* in a polluted river and municipal wastewater treatment works, found that this yeast species prefers oxygen limited conditions (Biedunkiewicz and Ozimek, 2009; Stone et al., 2012). It is therefore possible that, *C. albicans* may not have survived the preparation of the formulated influent or the largely aerobic environment in the rhizofiltration system.

The planted and unplanted sides may offer selective conditions favouring the survival of different *Candida species* in the respective sides of the system. Using next generation sequencing we found that the relative abundance of *Candida* OTUs was very low in the planted sand fractions. Interestingly, *Candida tropicalis* was one of the *Candida* OTUs identified and was also one of the most frequently isolated PPY strains in this study. In the unplanted side, genetic evidence of *Candida* species was below the detectable limit.

This study provided evidence supporting the hypothesis that the rhizofiltration system plants may have selected for a fungal community different from that of the unplanted sand. Unfortunately, the majority of the OTUs could not be identified to ranks lower than the phylum. In the unplanted sand most OTUs were found to represent unidentified fungi (Figure 4.6). These findings highlight how vastly unexplored fungal communities are in engineered environments. All the ascomycete OTUs that were identifiable to a lower rank belonged to the Pezizomycotina. Genetic evidence for the presence of members of the Saccharomycotina, which include the genus *Candida,* was generally below the detectable limit. Species of the Saccharomycotina are mostly found in nutrient rich environments where carbon in the form simple sugars occur abundantly (Blackwell et al., 2009). The carbon trapped in the rhizofilter medium was probably recalcitrant, favouring the growth of

basidiomycetes which are generally capable of metabolizing more complex compounds such as chitin, lignin and tannins (Botha, 2006; Walker, 1998).

The only abundant identifiable yeast species was the basidiomycete *Trichosporon ovoides*. This yeast had a higher relative abundance in the planted than the unplanted side. *Trichosporon* species are widely distributed in the environment but can also occur in the human gastrointestinal tract, oral cavity, respiratory tract and on the skin (Colombo et al., 2011). Moreover, strains of *Trichosporon* have been reported to be capable of causing deep seated mucosal infections in immunocompromised individuals, as well as superficial infections and allergic pneumonia in immunocomponent hosts (Colombo et al., 2011).

5 Conclusions

This study showed that PPY, able to grow at 37 °C, could be removed from a formulated influent representing polluted urban runoff using rhizofiltration. The PPY species in the formulated influent correlated with chemical indicators of faecal pollution. These chemicals also serve as nutrients for the fungal community in the rhizofilter medium, which may have selected for specific fungal species. This caused the PPY community composition in the effluents of both the planted and unplanted sides to differ from the formulated influent. Additionally, this is the first study to show that wetland plants in an engineered ecosystem selects for a specific yeast community. The fungal community of the planted sand was dominated by basidiomycetes and the less abundant ascomycetes were mostly members of the Pezizomycotina. The conditions in the rhizofiltration system appeared to prohibit the proliferation of potentially pathogenic *Candida* species to levels higher than that found in the formulated influent. However, this study found evidence that *Candida* species could survive in low numbers within the filter medium. Nevertheless, rhizofiltration could help to

break the faecal oral route of certain *Candida* spp. and possibly infections, particularly in areas with large populations of HIV infected individuals, lacking sanitation and water supply infrastructure.

6 References

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Figures and tables

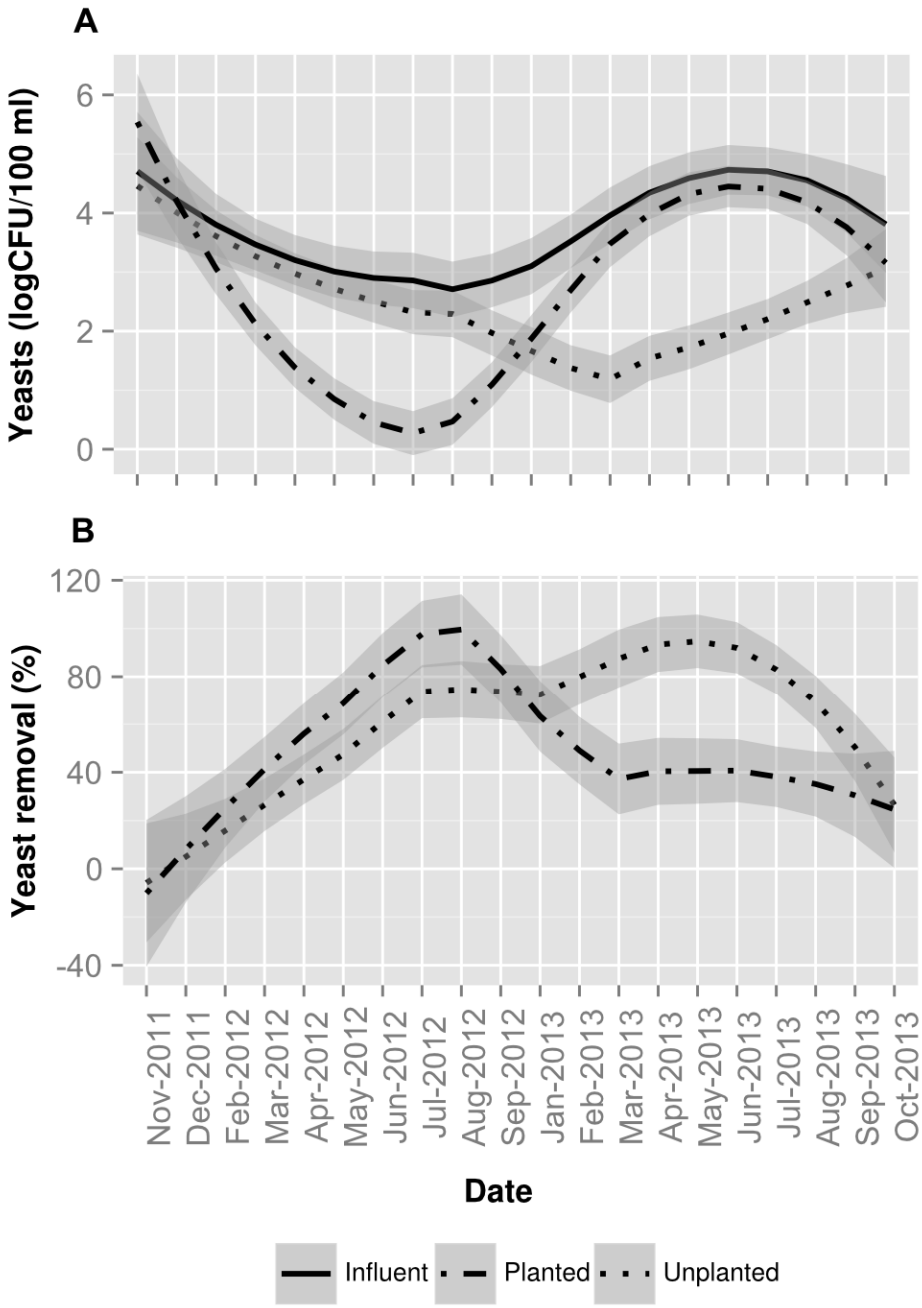

Figure 4.1: Potentially pathogenic yeast concentrations (A) and removal (B) after the formulated influent (resembling urban effluent) had passed through the planted and the unplanted side of the rhizofiltration system. Curves were smoothed with the LOESS non-parametric local regression statistic and shaded areas indicate a 95 % confidence interval.

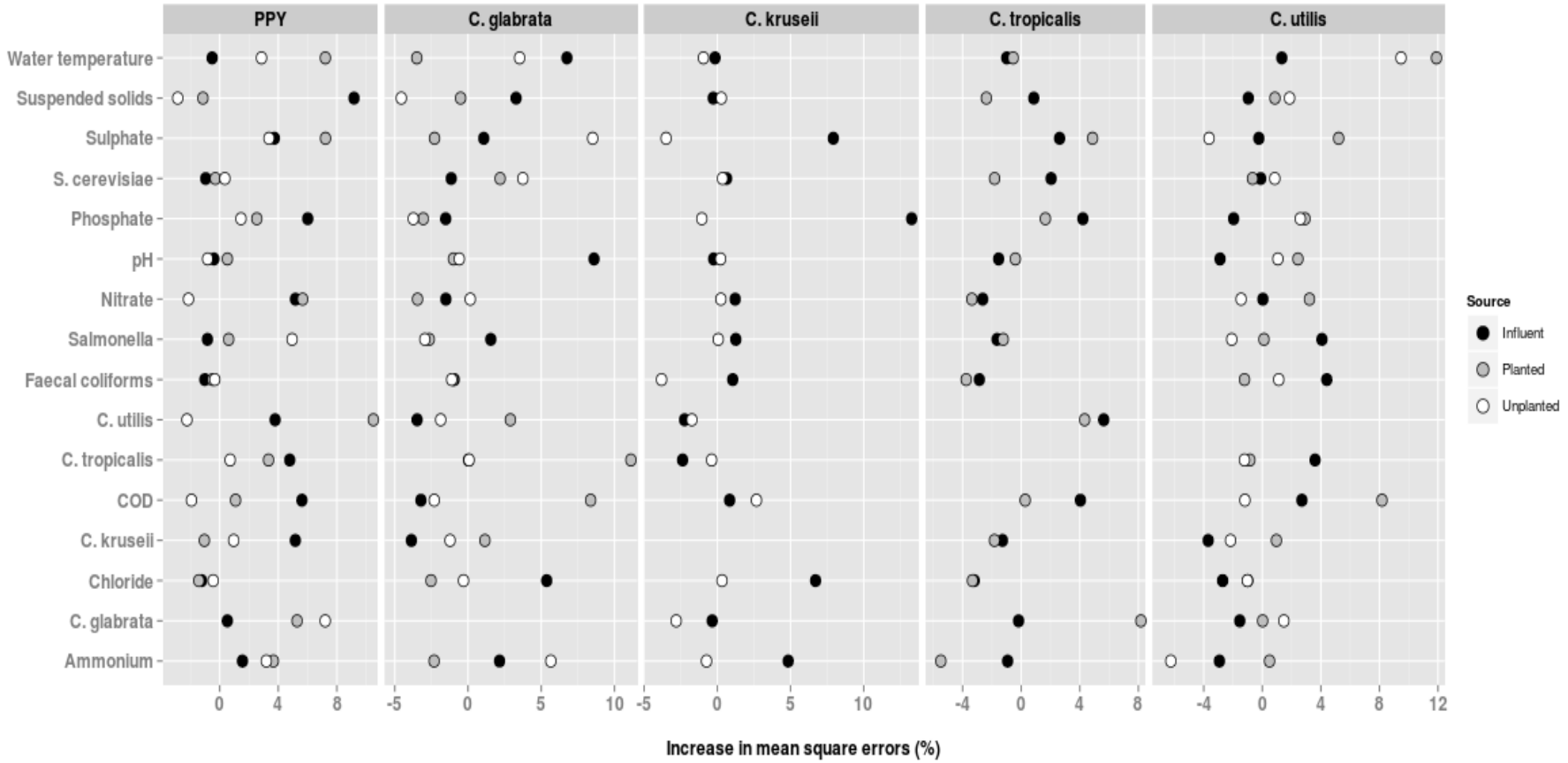

Figure 4.2: Variable importance plots for predictor variables from random forests regression used for predicting the concentration and relative abundance of potentially pathogenic yeasts (PPY). The increase in means square errors is the average increase in squared out of bag residuals when the variable is permuted. Higher values of increase in mean square errors indicate variables that are more important in the regression.

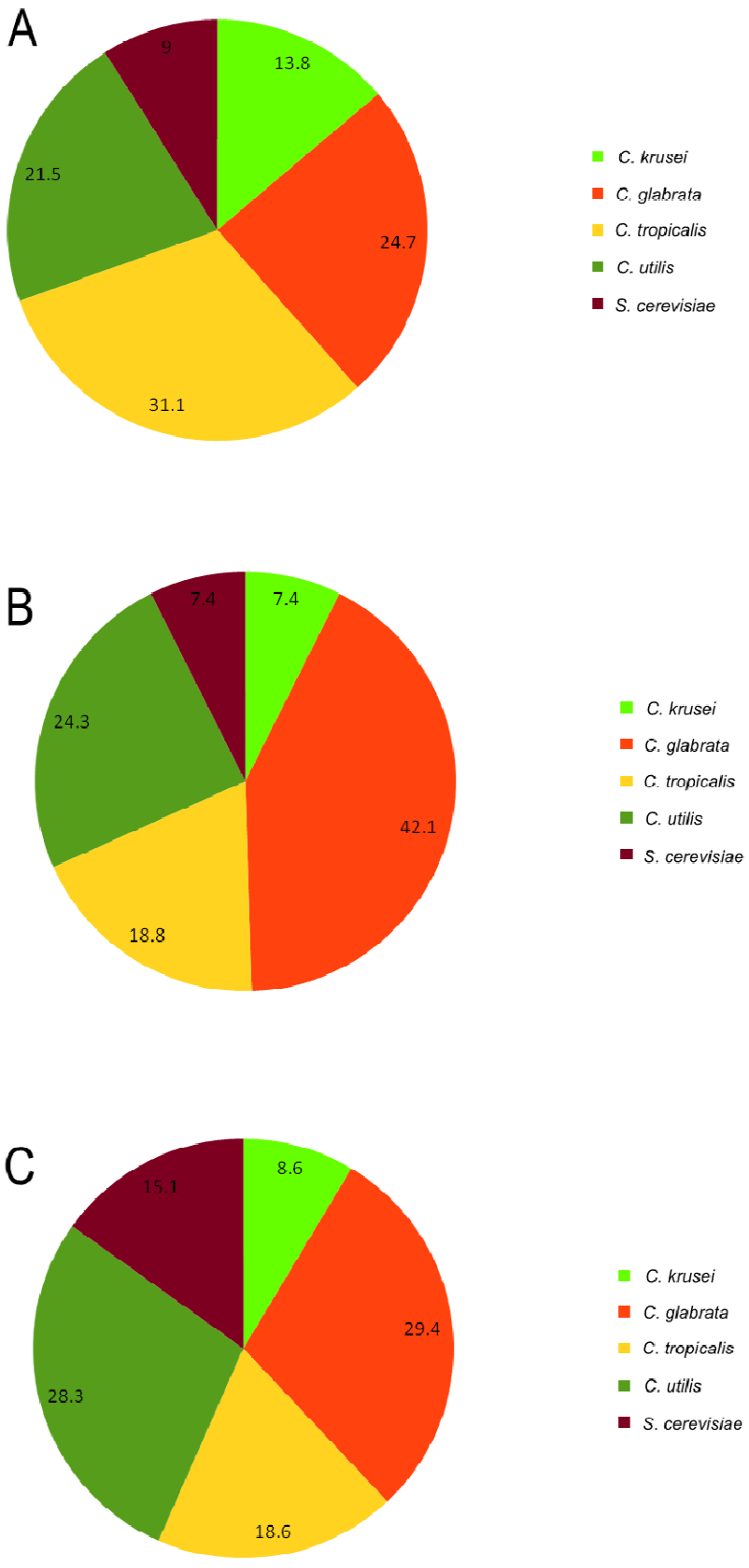

Figure 4.3: Relative abundance (%) of potentially pathogenic yeasts which occurred consistently in the wastewater across 20 Monthly sampling events from November 2011 to October 2013. A = Influent; B = Unplanted effluent; C = Planted effluent

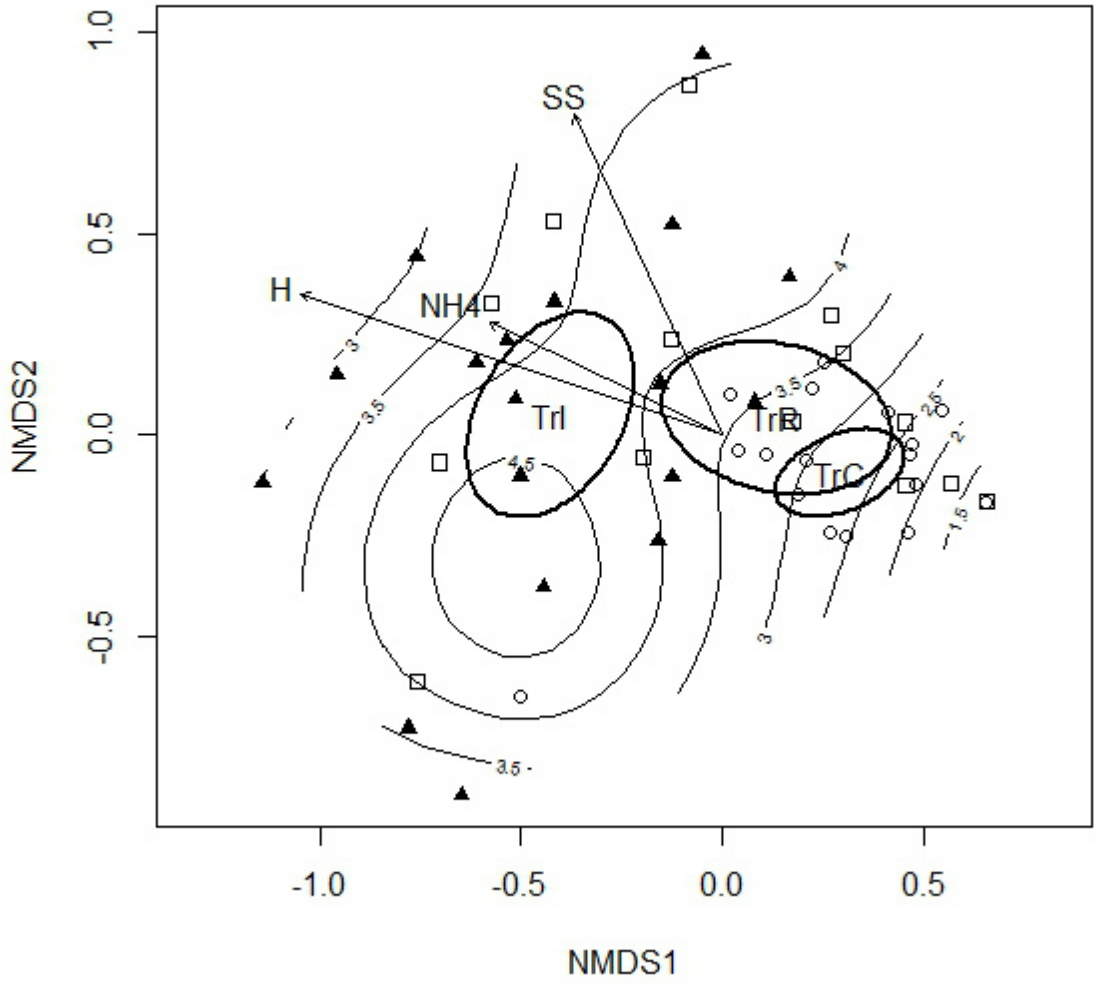

Figure 4.4: Non-metric multi dimensional scaling (NMDS) ordination plot of potentially pathogenic yeast community data (stress = 0.14), indicating significant (p = 0.001) differences in community composition in the formulated influent (TrI), planted side effluent (TrR) and unplanted side effluent (TrC). Circles = unplanted effluent, Squares = planted effluent, Triangles = formulated influent. Ellipses indicate standard error from point averages with a confidence limit of 0.95. Arrows of vectors points to the direction of most rapid change in the environmental variable, or the direction of the gradient, and the length of the arrow is proportional to the correlation between ordination and variable. Goodness-of-fit of vectors (r^2): Shannon's diversity index (H) = 0.381 ($p < 0.0001$); suspended solids (SS) = 0.241 ($p < 0.0001$); NH_4^+ = 0.129 ($p< 0.05$). Contours indicate the potentially pathogenic yeast concentration (log CFU.100 ml^{-1}).

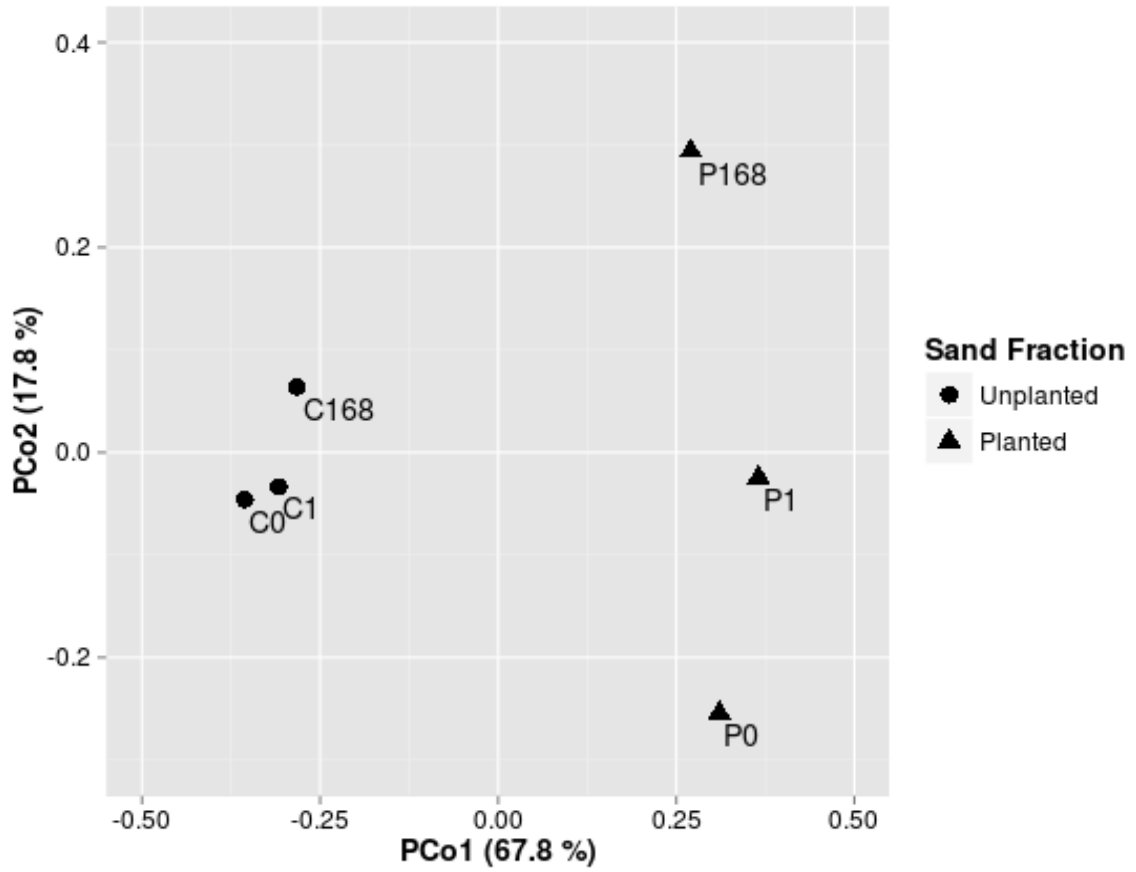

Figure 4.5: Principal coordinate analysis of the generalized UniFrac alfa 0.5 distance matrix showing a significant difference (GPERMANOVA F-model = 11.08 ; p = 0.044) in fungal community composition between the planted and unplanted sides of the rhizofilter. Numbers indicate the sampling times: 0h before, 1h and 168h after wastewater was released onto the rhizofilter.

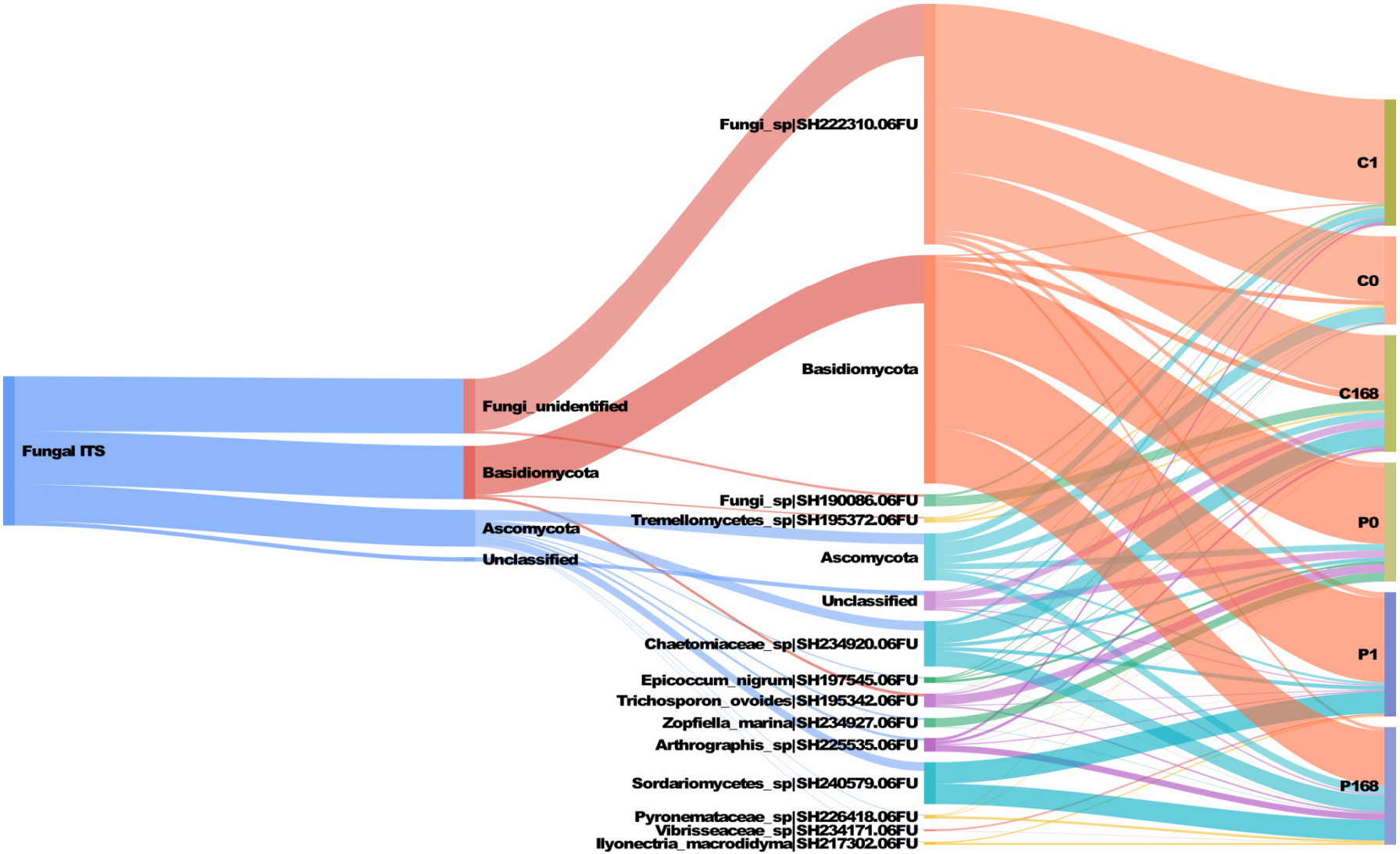

Figure 4.6: Relative abundance of fungal OTUs in the total dataset and in each of the six samples. C= unplanted controls side, P=planted side (experiment) of the rhizofilter.

Table 4.1: Means and standard deviations of potentially pathogenic yeast (PPY), physical and chemical data from twenty sampling events occurring monthly from November 2011 until October 2013.

Variable	Influent	Effluent		Removal (%)	
		Planted (experiment)	Unplanted (control)	Planted (experiment)	Unplanted (control)
PPY (log CFU.100ml^{-1})	3.7 ± 1.5 (113)	2.7 ± 2.2$_+$**** (228)	2.4 ± 1.7 (227)****	50.4 ± 62.8 (228)	61.5 ± 55.5 (227)
pH	6.5 ± 0.5 (64)	6.1 ± 0.6**** (126)	6.1 ± 0.6 (126)****	N/A	N/A
T (°C)	19.6 ± 5.8 (64)	17.6 ± 5.4**(122)	18.2 ± 5.1 (122)	N/A	N/A
NH_4^+ (mg.L^{-1})	65.7 ± 86.8 (19)	18.3 ± 12.4** (38)	19.4 ± 12.8 (38)**	55.2 ± 27.4 (38)	52 ± 29.1 (38)
NO_3^- (mg.L^{-1})	4.2 ± 15.5 (19)	22.2 ± 37 (38)	18.4 ± 35.7 (38)	-52.1 ± 56.1 (26)	-11 ± 77.5 (26)
PO_4^{3-} (mg.L^{-1})	14.8 ± 15.9 (20)	13.7 ± 11.2 (36)	11.1 ± 10.7 (36)	1.9 ± 41.1 (36)	13.9 ± 36.2 (36)
SO_4^{2-} (mg.L^{-1})	236.6 ± 468.6 (19)	58 ± 85.24(38)	48.2 ± 97 (38)	-18.6 ± 74.4 (38)	-12.4 ± 74.4 (38)
Cl^- (mg.L^{-1})	96 ± 29.1 (19)	90.73 ± 23.5 (38)	90.7 ± 33.4 (38)	4.6 ± 14.3 (38)	6.8 ± 13.1 (38)
COD (mg.L^{-1})	661.2 ± 336.4 (17)	451.5 ± 334.1* (38)	448.7 ± 340.4* (38)	33.1 ± 25.1 (38)	35.3 ± 25.4 (38)
SS (mg.L^{-1})	139.6 ± 114.4 (17)	56.9 ± 47.3** (38)	61.1 ± 40.1 (38)**	37.5 ± 48.2 (38)	40.9 ± 45.2 (38)

Kruskal-Wallis ANOVA significant difference (p) values: < 0.05 = *, < 0.01 = **, < 0.0001 = ****, *significantly different from influent, $_+$significantly different from unplanted control. Numbers in parenthesis = N data points. T = water temperature, COD = chemical oxygen demand, SS = suspended solids.

Table 4.2: Significant Spearman rank order correlations (r-value) among potentially pathogenic yeasts (PPY) concentration, PPY removal (PPY-R) and the physico-chemical parameters.

Variables	Unplanted (Control)		Planted (Experiment)		Influent
	PPY	PPY-R	PPY	PPY-R	PPY
Influent PPY	0.114	**0.474***	**0.822****	**-0.466***	-
Influent T	0.324	-0.427	0.232	**-0.651****	-0.033
Influent PO_4^{3-}	0.270	0.178	**0.518***	-0.387	**0.668****
NO_3^-	0.308	-0.363	0.376	**-0.601****	NA
Influent NO_3^-	-0.002	0.022	0.286	**-0.560***	**0.509***
NH_4^+	0.305	-0.195	**0.536***	-0.379	NA
Influent NH_4^+	0.332	-0.154	**0.467***	-0.363	-0.107
Cl^--R	-0.339	**0.614****	-0.043	0.286	NA
SO_4^{2-}	-0.137	-0.007	0.218	**-0.486***	NA
Influent SO_4^{2-}	-0.080	0.152	0.272	-0.415	**0.571***
Influent COD	0.072	0.188	0.202	-0.205	**0.499***
SS-R	0.433	**-0.586****	-0.034	0.118	NA
Influent-SS	**0.510***	**-0.560***	-0.178	0.167	**-0.496***

*p<0.05; **p<0.01, T = water temperature, R = removal, COD = chemical oxygen demand, SS = suspended solids.

Table 4.3: Dominant yeasts isolated during November 2011 to October 2013 from the formulated influent and effluent of both the unplanted (control) and planted (experiment) sides of the rhizofilter.

	Influent		Unplanted		Planted	
Species	**No. of strains**	**Percentage of total**	**No. of strains**	**Percentage of total**	**No. of strains**	**Percentage of total**
C kruseii	43	13.8	15	7.4	24	8.6
C. glabrata	77	24.7	85	42.1	82	29.4
C. tropicalis	97	31.1	38	18.8	52	18.6
C. utilis	67	21.5	49	24.3	79	28.3
S. cerevisiae	28	9.0	15	7.4	42	15.1
Total	312	100	202	100	279	100

Table 4.4: Mean and standard deviation of the diversity, richness and evenness of the fungal communities in the planted and unplanted sides of the rhizofiltration system. No significant difference (Kruskal Wallis-ANOVA $p>0.05$) was observed between the planted (experiment) and unplanted (control) side in any of the indices.

Alfa-diversity indices	**Unplanted (control)**	**Planted (experiment)**
Shannon diversity	3.5±0.28	3.6±0.02
Inverse Simpson diversity	16.5±6.36	15.4±2.05
Fishers alpha diversity	20.2±3.16	29.2±5.08
Richness	110.7±14.29	149.3±21.22
Pielou's Evenness	0.7±0.05	0.7±0.02

Chapter 5

1 General conclusions and future research

The treatment of polluted urban runoff before it enters river systems is of critical importance to protect the hydrosphere. Globally, various treatment options collectively called best management practices (BMPs) were evaluated to alleviate this pollution (Granato, 2014). The BMPs often mimic the natural ecosystem services provided by riverine and estuarine ecosystems (Karr and Schlosser, 1978; Naiman and Decamps, 1997). Studies on BMP performance mainly focused on the empirical determination of pollutant removal and black box modelling of treatment processes. However, these models are over simplifications of such treatment processes (Olszewski and McCuen, 2014). On the other hand process models attempt to describe BMP behaviour by quantitatively representing the actual processes within a wetland. Although it is recognized that microbial populations play an important role in these pollutant removal processes, limited fundamental theories exist on how microbial populations interact with their physico-chemical environments within BMPs (Werker et al., 2002).

With this study we aimed to acquire knowledge on microbial processes involved in pollutant removal from urban effluent via rhizofiltration, a low impact development best management practice (LID-BMP). To effectively integrate new knowledge garnered in this study with current literature we firsts constructed a conceptual model of treatment processes that may occur within the rhizofilter (Chapter 1). This also allowed us to identify gaps in the existing knowledge of pollutant treatment mechanisms in LID-BMPs. The focus of the conceptual mode, and the subsequent experimental work conducted during this study was on the removal of nutrients, enteric bacteria and potentially pathogenic yeasts (PPY) from simulated urban effluent. The key findings of this study were added to the conceptual model (Figure 5.1).

We found that the rhizofilter could remove pollutants such as ammonia (NH_4^+), COD, coliphages, faecal coliforms, PPY belonging to the genus *Candida*, phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), suspended solids as well as bacteria belonging to the genera *Salmonella* and/or *Shigella*. The sorption capacity of the rhizofilter was determined for each of the measured pollutants except for PPY. We found that the filter medium was rapidly saturated with most of the pollutants (Chapter 2). Therefore, pollutant removal was generally limited by the physical sorption capacity of the sand and rock filter media. Consequently, the effluent concentrations of the pollutants were mostly above the range of regulatory requirements (DWAF 1996).

Greater pollutant removal can be achieved with so called treatment trains (Bastien et al., 2010). Therefore, it is envisaged that the rhizofilter will be used as a first or second barrier in a BMP treatment train. Structural BMP treatment trains can improve both the water quality as well as total runoff volume of the final effluent, thereby minimizing the effects on receiving waters. Individual structural BMPs may remove pollutants through several mechanisms. However, the most effective treatment trains are constructed from BMPs with different dominant pollutant removal mechanisms, such as integrating of filtration (e.g. rhizofiltration) with sedimentation (e.g. wet retention pond) (Bastien et al., 2010).

A defining feature of the rhizofilter was the presence of the wetland plants *Typha capensis* and *Phragmites australis*. It was previously demonstrated that these plants altered the physical characteristics of the rhizofilter medium, which resulted in a higher hydraulic conductivity compared to the unplanted control filter medium (Wilsenach et al., 2014). Also, it was hypothesized that the plants play a pivotal role in pollutant removal processes by providing a physico-chemical environment which harbours a more active and resilient microbial community (Wilsenach et al., 2014). Using metagenomics and phospholipid fatty

acid analysis we found evidence supporting the hypothesis that wetland plants have a significant effect on the microbial communities in engineered ecosystems (Chapter 3). However, the difference in microbial community composition and the presence of the plants did not result in a significant increase in pollutant removal compared to an unplanted control.

The importance of plants in pollutant removal processes will likely be greater in BMPs with longer hydraulic retention times. The diffusion coefficient of oxygen in water is a factor of 10^4 lower than in air, rendering wetland soils anoxic almost immediately after flooding (Brune et al., 2000). In such anaerobic conditions plants may help to improve the redox potential by releasing oxygen into the filter medium through their roots (Brown et al., 2000). In the rhizofilter however, evidence of nitrification and COD removal suggest that oxidative conditions prevailed in both the unplanted and planted sides of the rhizofilter. The impact of oxygen release from plant roots on the redox state of the filter medium was probably minimal. Therefore, in this system and other similar designs, the rapid percolation rate and intermittent loading of pollutants may be the dominant factors creating conditions favourable for aerobic bacteria. Also, the main value of microbial populations in this particular system seemed to be the biological regeneration of the filter media by mineralizing trapped pollutants (Chapter 2). Due the rapid percolation rate of the rhizofilter biological removal of pollutants from the wastewater as it flowed through the rhizofilter was limited.

Analysis of microbial community composition within the rhizofilter revealed that both the planted and unplanted sides of the rhizofilter selected for actinomycetes (Chapter 3). This may have been a consequence of the aerobic nature of the filter medium and/or the high amounts of recalcitrant carbon loads released onto the rhizofilter. The type of organic

carbon in wastewaters may be an important determinant of microbial community composition and should be taken into consideration in treatment process modelling. Apart from carbon, other differences in nutrient composition, such as the carbon:nitrogen ratio, or presence of xenobiotics in urban effluent may affect microbial community structure and diversity within the rhizofilter (Crenshaw et al., 2002; Nogaro et al., 2007; Zhu and Chen, 2001). This implies that stochastic variations in urban effluent nutrient composition may influence pollutant removal efficiency. In the rhizofilter the plants appeared to attenuate the temporal variation in microbial community structure compared to the unplanted control, however this did not translate into more consistent pollutant removal.

Changes in microbial community composition, as was observed in the unplanted control side of the rhizofilter (Chapter 3), do not always correspond to changes in functional redundancy. A measure of functional redundancy is the number (richness) of different species that perform a specified function or their diversity (Yin et al., 2000). In this study we found that the total diversity of the unplanted and planted sides were similar, however it would be interesting to determine whether there are differences in the richness or diversity in functional groups. These functional groups can be defined by their ability to utilize nitrogen or carbon compounds expected to occur in urban effluent.

Network analysis revealed that genetic evidence of microbial communities in the rhizofilter correlated with indicators of microbial activity (PLFA analysis) and chemical measurements (Chapter 3). This evidence supports the theory that bacterial populations play a significant role in pollutant removal processes. In addition, the metagenomic analysis was reliable in describing the population dynamics. We could also identify possible important role players, such as the actinomycetes, in the pollutant removal processes which may be of value in future process modelling efforts. Furthermore, this

correlative approach allowed us to identify general trends from which hypotheses were derived for future research.

One such hypothesis is that closely related bacteria co-occur so that they may take advantage of shared phenotypic characteristics, manipulated by gene transfer or signalling mechanisms, to affect a cascade of reactions through which complex carbon sources are completely mineralized. Such cascades would be beneficial in pollutant removal processes and could perhaps be quantified through a single genetic or metabolic pathway that encompasses the phylogenetic diversity. Providing evidence to support this hypothesis would require techniques different from those used in this study. Although PLFAs are useful in describing changes in activity of broad groups of bacteria, it does not take into account the immense genetic diversity and potential of microbial populations. Genetic evidence of microbial lineages on the other hand is difficult to quantitatively reconcile with changes in process chemistry. A closer approximation of biological pollutant removal process rates could possibly be made by studying the genetic potential through the metatranscriptome present in the rhizofilter media.

The short half -life of mRNA in the environment, compared to DNA, ensures that only the very recently active biological components are measured (Dubelman et al., 2014; Wang et al., 2012). In addition, metatransricptomic analysis can provide insight into gene regulation of microbial communities which may include archaea, eubacteria, fungi, and unicellular animals within BMPs (Yu and Zhang, 2012). This can be combined with measurements of compounds liberated during pollutant treatment, for example reduced and oxidized states of nitrogen, carbon, or other volatile organic compounds that may be related to specific metabolic pathways. Alternatively, a chemical marker could be incorporated into cell biomass which can be used to discriminate between active and senescent microbial

populations. Such efforts may allow us to elucidate the fundamental organizing principals dictating community composition and its relation to pollutant removal.

However not all pollutant removal mechanisms can be elucidated by analysing the metatranscriptome. This is especially true for the removal of pathogens which may be subjected to interspecies competition or cooperation on various trophic levels. Additionally, many pathogens may not be recognized by the current techniques used to monitor microbial contaminants in the environment. This study may serve as an example of the unexpected consequences BMP designs might have in terms of the removal of microbial contaminants.

In the rhizofilter, the plants created conditions ideal for the proliferation of the Mycobacteriaceae (Chapter 3). Species representing the Mycobacteriaceae which occur in soil were shown to degrade anthropogenic organic compounds such as paraffinic hydrocarbons, benzenoic acid and phenol (Bushnell and Haas, 1941; Dean-Ross et al., 2002). This capability to metabolize such compounds is useful in treating urban runoff which may be a rich source of such compounds. However species of the Mycobacteriaceae notably *Mycobacterium tuberculosis* cause severe pulmonary infections and it is highly prevalent in South Africa among individuals suffering from HIV/AIDS (Crowther-Gibson et al., 2014; Govender et al., 2010). Although the Mycobacteriaceae identified in this study are most commonly found in soil they may be opportunistic pathogens. Research on the virulence of the *Mycobaterium* population in the rhizofilter and other BMPs may give us insight on the dissemination of *Mycobacterium tuberculosis* and closely related opportunistic pathogens in the environment.

The oxidative state within the rhizofilter that contributed the selective conditions for aerobic actinomycetes was not conducive for the survival of commensal gut inhabiting bacteria or pathogens that are mostly anaerobes of facultative anaerobes (Tamplin, 2003). The metagenomic analyses showed that despite the high counts of faecal indicator bacteria on MacConckey agar, the relative abundance of the Enterobacteriaceae within the rhizofilter was very low (Chapter 3). Instead operational taxonomic units (OTUs) of other gut commensals namely the Campylobacteraceae, Moraxellaceae, Porphyromonadaceae and Prevotellaceae were more frequently detected and had a higher relative abundance although it was still less than 1 % of the total bacterial OTUs. Also, it was shown by others that facecal indicator bacterial numbers do not always correlate with the presence of pathogens (Staley et al., 2012). Therefore using selective media to enumerate only members of the Enterobacteriaceae in natural environments may cause an underestimation faecal pollution risk.

Apart from the enteric bacteria, opportunistic pathogenic yeasts could occur in urban effluent. In this study we investigated the occurrence and removal of PPY from wastewater (Chapter 4). We found that rhizofiltration was effective in the removal of PPY however certain potentially pathogenic *Candida* species could survive in the rhizofilter. The dissemination of these yeasts in the environment is relevant in the context of the large immunocompromised population in South Africa. Candidiases is one of the most prevalent yeast infections with the species isolated in this study the causative agents in many clinical cases (Crowther-Gibson et al., 2014; Kreusch and Karstaedt, 2013; Singh et al., 2002).

Metagenomic analysis of the fungal populations in the rhizofilter showed that PPY belonging to the genus *Candida* had a low relative abundance (Chapter 4). However, the infectious dose of *Candida* species is unknown and even low concentrations may put

immunocompromised people living near polluted water sources at risk. Future studies comparing the virulence of environmental strains to clinical isolates may prove valuable in determining the risk that these yeasts pose to people living in settlements with poor sanitation infrastructure. Notwithstanding the occurrence of potential pathogens, most fungi are saprotrophs (Evert and Eichhorn, 2013) and may be a key part of the cascade of processes that might occur in the degradation of carbon in polluted water. Therefore, the interaction between fungi and other life forms and their potential use in pollutant removal technologies should be investigated in future.

We believe that the information presented in this study will aid in the development of more informed BMP designs that will not only focus on the removal of pollutants such as nutrients but also potential pathogens. Furthermore, investigating the rhizofilter advanced our knowledge on the microbial processes that might occur in natural ecosystems subjected to high pollutants loads.

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Figure

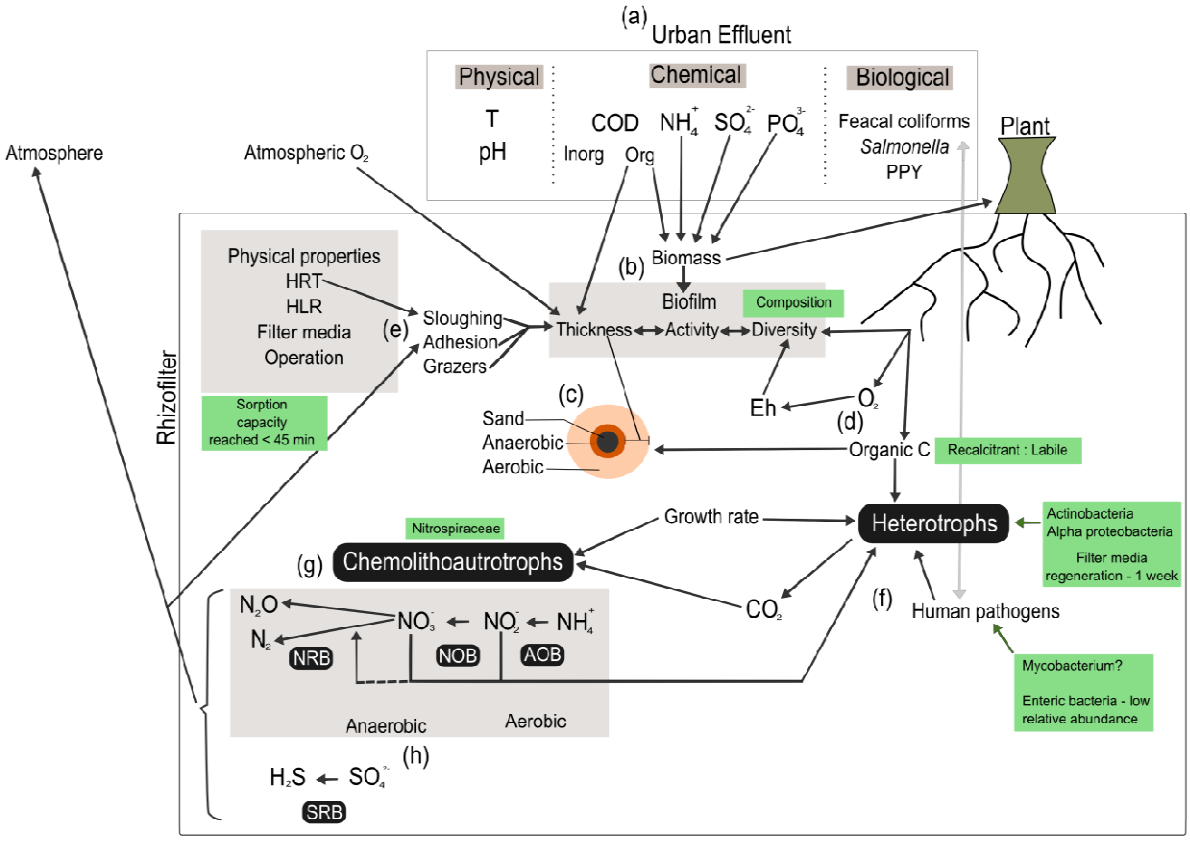

Figure 5.1: Conceptual model of treatment processes which may occur in the rhizofiltration system with information garnered in this study shown in green boxes. (a) Urban effluent contains numerous pollutants but the focus of this study was on selected nutrients and microbial indicator organisms. Pollutant removal is hypothesized to be mediated by microbial biofilms (b) on surfaces such as the sand media (c) and plant roots (d) within the predominantly aerobic rhizofilter matrix. Biofilm activity is a function of biofilm thickness diversity and composition which is influenced by the redox potential (Eh), physical properties such as the sorption capacity (e) of the system and plant root exudates (d). The effect of oxygen release by the plant root may be negligible due the high aeration rate of the filter media. Organic carbon from the urban effluent (COD) and the ratio of recalcitrant to labile compounds (d) promotes and selects for a heterotrophic community with a high relative abundance of actinomycetes and α-Proteobacteria (f). Respiration by the heterotrophs produces CO_2 that is used as an inorganic carbon source by chemolithoautotrophs (g) which are responsible for nitrogen (nitrification by Nitrospiraceae) and sulphur cycles in the system (h). The products of the nutrient cycles could also influence biofilm characteristics. The biomass ratio between these two groups of bacteria is a function of their respective temperature dependant growth rates. Redox gradients in biofilms (c) and the rhizosphere (d) exist due to microbial respiration and differential spatial and temporal release of oxygen by the plant roots. This may allow for both aerobic and (restricted) anaerobic pollutant removal processes. All bacterial pathogens are heterotrophs (f) and thus, at some stage, form part of the heterotroph community in the rhizosphere. Enteric bacteria and potentially pathogenic yeasts had a low relative abundance and did not proliferate in the filter media. However potential pathogens within the genus *Mycobacterium* may occur in the rhizofilter.

Abbreviations: T = temperature; COD = chemical oxygen demand; Org = organic; HRT = hydraulic retention time; HLR = hydraulic loading rate, C = carbon; NRB = nitrate reducing bacteria; NOB = nitrite oxidizing bacteria; AOB = ammonia oxydizing bacteria; SRB = sulphate reducing bacteria; PPY = potentially pathogenic yeasts.

Supplementary material

Chapter 3

1 PLFA extraction

Four grams of lyophilized sand were weighed into a 30 ml centrifuge tube. Subsequently 3.4 ml of 0.1 M phosphate buffer (pH 7.0), 4 ml chloroform and 8 ml MeOH was added. Tubes were placed horizontally in an orbital shaker for 2 h at 200 rpm. After centrifugation for 10 min at 2500 rpm (Beckman Coulter Avanti J-E centrifuge with Beckman JA 14 rotor), the liquid phase was decanted into a 30 ml test tube with a Teflon lined screw cap. Phosphate buffer (3.6 ml) and 4 ml chloroform was added to the sample. The tubes were vortexed for 5 s and subsequently left overnight in the dark at 4 °C for phase separation. The upper phase was removed by aspiration and discarded while the lower chloroform phase, containing the extracted lipids was evaporated under nitrogen. Subsequently, the lipids were resuspended and transferred to a clean test tube using 2 ml chloroform and dried. Lipid classes were separated by solid phase extraction (SPE) using 6 ml silica SPE cartridges (bed weight, 500 mg; Supelclean LC-Si) that were each conditioned with 3 ml methanol followed by 3 ml chloroform. Lipids, suspended in chloroform, were consecutively transferred to the SPE cartridge in four 250 µl aliquots, and allowed to pass through the silica. After washing the cartridge with 5 ml chloroform, followed by 10 ml acetone, phospholipids were eluted with 5 ml methanol. Subsequent to evaporation under nitrogen the phospholipids were transesterified to fatty acid methyl esters (FAMEs), using the mild alkaline KOH method, and extracted into 4 ml of hexane. The FAMEs were analyzed by tandem gas chromatography- mass spectrometry (GC-MS) using an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) and a Hewlett Packard 5973 mass spectra detector (MSD). FAMEs were identified by comparison of their mass spectra with those of 20 standards and mass spectra on the NIST05 atomic spectra database (National Institute of Standards and Technology, Gaithersburg, MD.) All chemicals used were chromatographically pure.

2 Modified primers

The modifications included addition of a PGM sequencing adaptor, a “GAT” spacer and a unique error correcting Golay barcode. A set of 23 uniquely barcoded forward primers and one reverse primer was used for unidirectional multiplex sequencing (Table S1). Amplicon product purification was performed via gel-electrophoresis using the e-gel system. The exact amplicon size distribution was determined using a 2100 Bioanalyzer (Agilent Technologies, USA). Amplicon library concentration was estimated with the Qubit 2.0 instrument using the Qubit dsDNA HS assay (Life Technologies). The concentration of each sample was adjusted to 10-15 pM and all samples were pooled. This pooled sample was used for emulsion PCR according to the Ion PGM™ 200 Xpress™ Template Kit manual (Life Technologies, Carlsbad, USA).

3 PCR conditions

PCR amplification was performed in a 15 µl reaction volume containing 7.5 µl 2x HiFi HotStart ReadyMix (Kapa Biosystems, Boston, MA, USA), 0.5 µM each of the forward and reverse primer and 50-200 ng DNA template. The PCR amplification conditions consisted of an initial denaturing step at 95 °C for 5 min followed by 35 cycles of 98 °C for 20 s, 75 °C for 15 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 1 min, whereafter the samples were held at 4 °C

4 Sequence analysis

Low quality reads were removed as follows: i) reads with no match to the forward primer barcode and adaptor sequences allowing one error, ii) reads not matching the PCR primers with at most three errors, iii) sequences with an average quality score of 25 or less iv) reads with a homopolymer length greater than 8 and iv) sequences which were shorter than 400 bp. Correctly detected primer sequences were trimmed off during this procedure.

Supplementary material

Chapter 3: Figures and tables

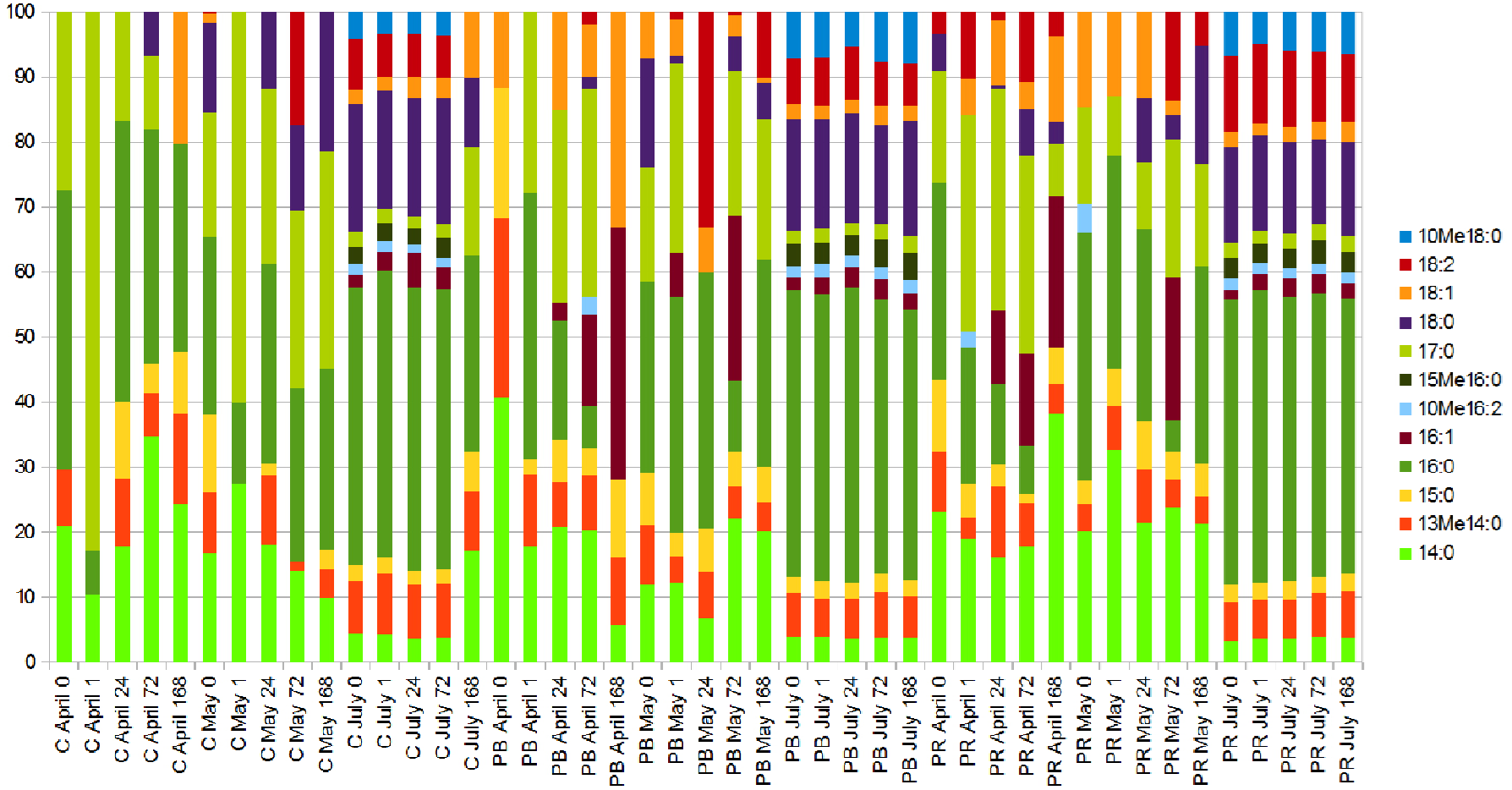

Figure S3.1: Relative abundance of phospholipid fatty acids. C=control, PB=planted bulk sand, PR=rhizosphere sand.

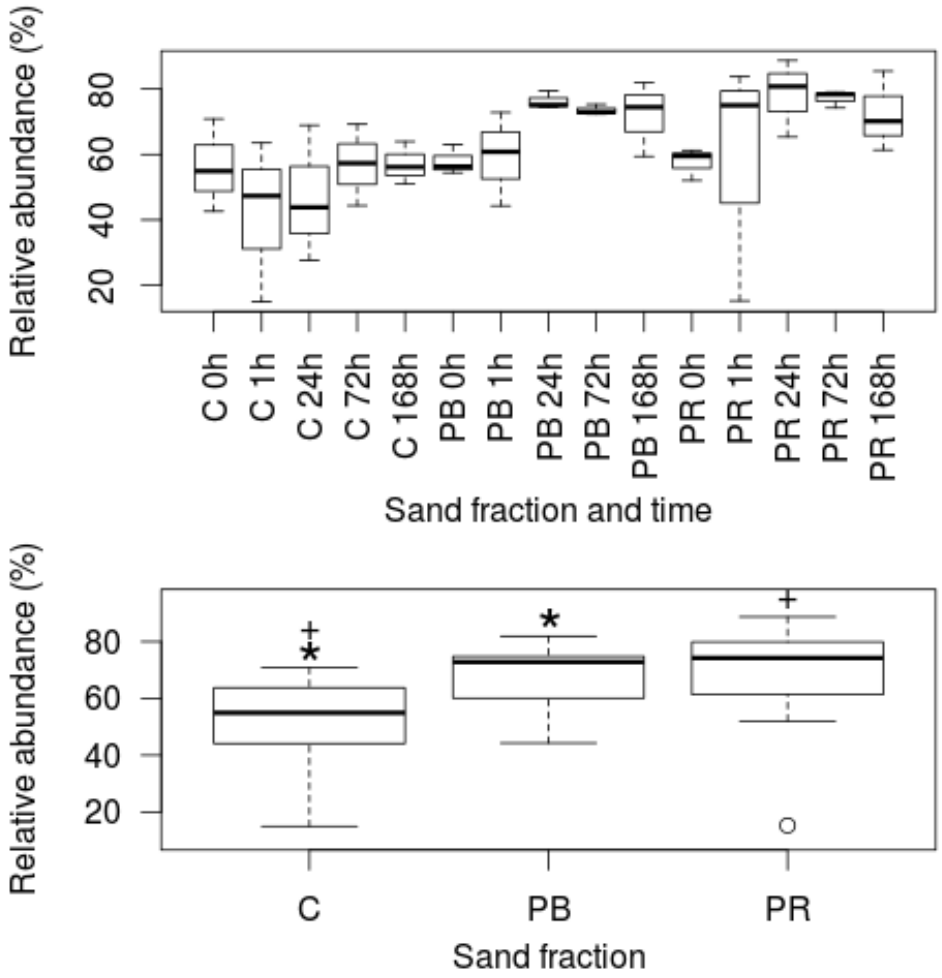

Figure S3.2: Boxplots of the relative abundance of the Actinomycetales before (0h) and after (1 -168 h) simulated urban effluent was added to the system and in the three sand fractions (C= unplanted control sand, PB=planted bulk sand, PR= rhizosphere sand.

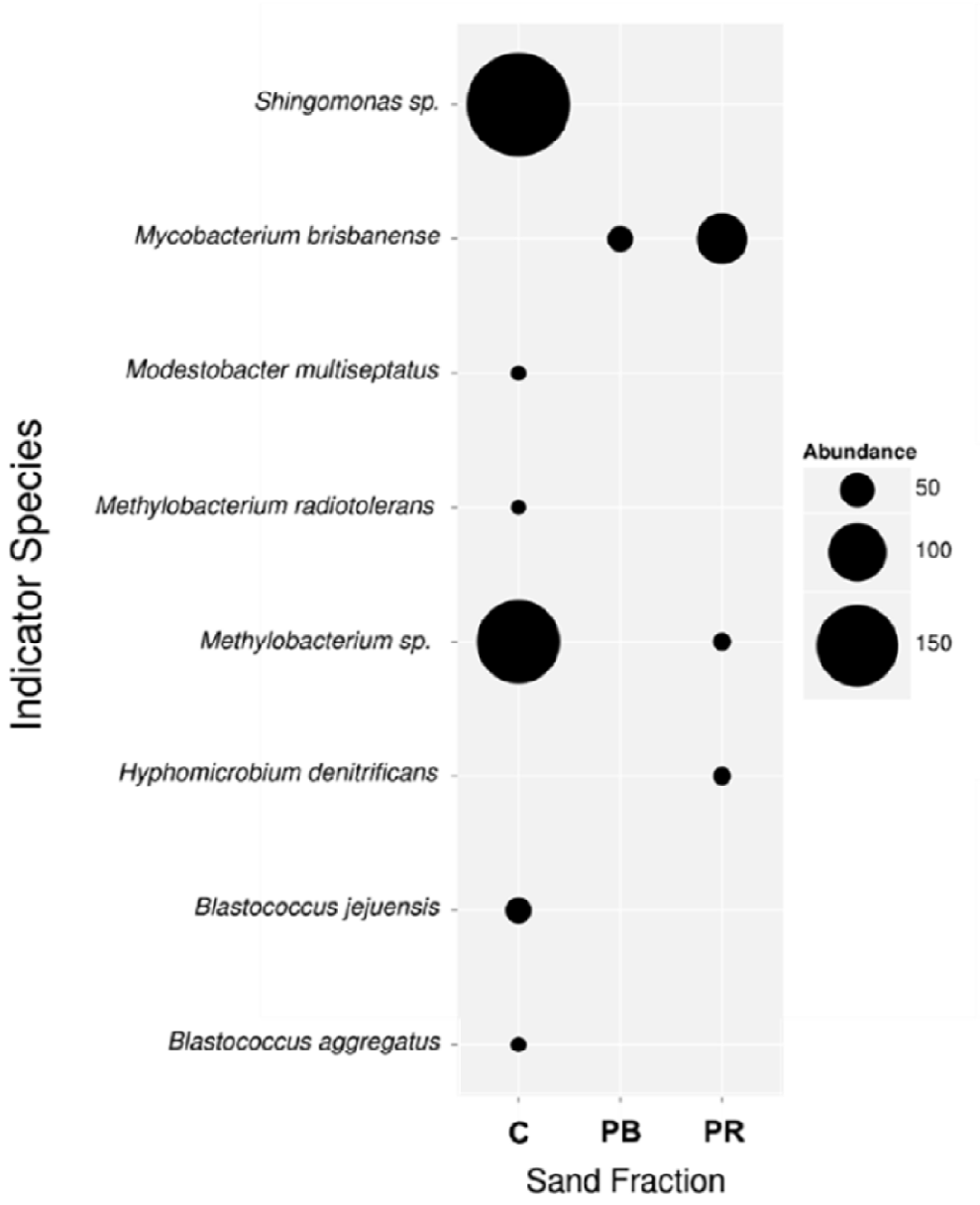

Figure S3.3: Highly abundant species which occurred frequently in the respective sand fractions and their relative abundance (number of sequences). Species were identified by selecting representative sequences from the indicator OTUs (MOTHUR indicator species algorithm) and aligning them to sequences on the NCBI database using the BLAST tool. C=unplanted control; PB=planted bulk sand; PR=rhizosphere sand

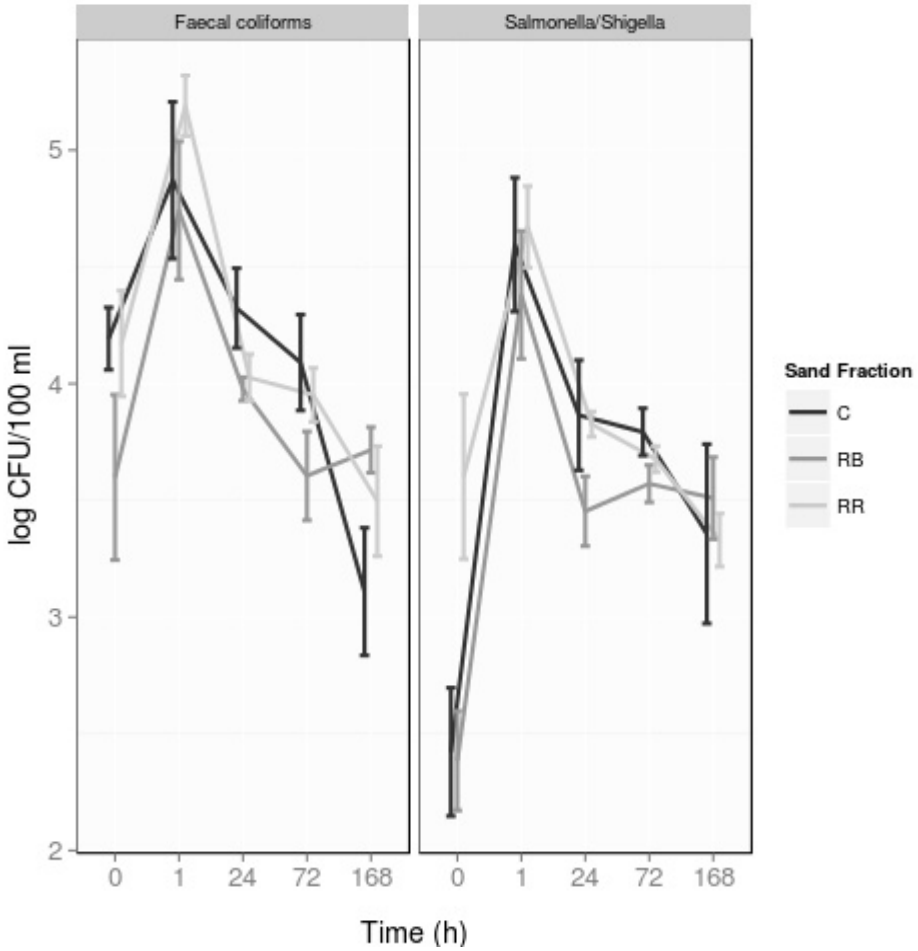

Figure S3.4: Change in faecal coliform and *Salmonella/Shigella* concentrations in the planted rhizofilter bulk (RB), rhizosphere (RR), and unplanted control (C) sand fractions before (0 h) and after (1-168 h) the addition of simulated urban effluent. Values are the mean of triplicate samples taken over a period three months. Whiskers indicate standard error of the means.

Table S3.1: Fusion primers used for ion torrent sequence analysis of the V4-V5 hypervariable regions of the 16S rRNA gene.

Name	Ion Torrent Linker Primer	Barcode	Spacer	16S rRNA gene Primer
Bacterial				
B1R	-	-	-	CTACCAGGGTATCTAATCCT
Bacterial				
B1F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAAGGTAAC	GAT	ACTCCTACGGGAGGCAGCA
B2F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAAGGAGAAC	GAT	ACTCCTACGGGAGGCAGCA
B3F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AAGAGGATTC	GAT	ACTCCTACGGGAGGCAGCA
B4F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TACCAAGATC	GAT	ACTCCTACGGGAGGCAGCA
B5F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAAGGAAC	GAT	ACTCCTACGGGAGGCAGCA
B6F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGCAAGTTC	GAT	ACTCCTACGGGAGGCAGCA
B7F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCGTGATTC	GAT	ACTCCTACGGGAGGCAGCA
B8F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTCCGATAAC	GAT	ACTCCTACGGGAGGCAGCA
B9F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGAGCGGAAC	GAT	ACTCCTACGGGAGGCAGCA
B10F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGACCGAAC	GAT	ACTCCTACGGGAGGCAGCA
B11F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCCTCGAATC	GAT	ACTCCTACGGGAGGCAGCA
B12F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAGGTGGTTC	GAT	ACTCCTACGGGAGGCAGCA
B13F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAACGGAC	GAT	ACTCCTACGGGAGGCAGCA
B14F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGAGTGTC	GAT	ACTCCTACGGGAGGCAGCA
B15F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTAGAGGTC	GAT	ACTCCTACGGGAGGCAGCA
B16F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTGGATGAC	GAT	ACTCCTACGGGAGGCAGCA
B17F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTATTCGTC	GAT	ACTCCTACGGGAGGCAGCA
B18F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGGCAATTGC	GAT	ACTCCTACGGGAGGCAGCA
B19F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTAGTCGGAC	GAT	ACTCCTACGGGAGGCAGCA
B20F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGATCCATC	GAT	ACTCCTACGGGAGGCAGCA
B27F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACCATCCGC	GAT	ACTCCTACGGGAGGCAGCA
B28F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCCGGAATC	GAT	ACTCCTACGGGAGGCAGCA
B29F	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGACCACTC	GAT	ACTCCTACGGGAGGCAGCA

a: Required fusion primers are generated by summation of all sequence columns in the 5'-3' direction

Table S3.2: Summary of sequence processing

Sample	Total reads	High quality reads	Non-chimeric, unique reads
1_0C	115061	11251	1403
1_0PB	166274	26556	906
1_0PR	204855	37228	1089
1_1C	80973	6797	861
1_1PB	238342	34247	1413
1_1PR	218005	34093	1128
1_2C	91656	8940	1133
1_2PB	244729	38452	1808
1_2PR	123787	16719	739
1_3C	116875	9158	1302
1_3PB	159730	23769	856
1_3PR	163536	28751	1102
1_4C	128776	11358	1577
1_4PB	109665	8294	1112
1_4PR	608848	49384	7281
2_0C	232509	37084	1407
2_0PB	310270	24971	3446
2_0PR	239727	37489	1301
2_1C	247138	38926	1385
2_1PB	269501	37518	1520
2_1PR	240196	38860	1732
2_2C	256345	40266	1809
2_2PB	222949	33843	1270
2_2PR	228110	34235	1657
2_3C	165080	29782	1196
2_3PB	238123	37338	1719
2_3PR	220591	37830	1710
2_4C	223270	33189	1096
2_4PB	285358	37615	1459
2_4PR	234443	37546	1809
3_0C	99549	11329	1611
3_0PB	101493	8409	1418
3_0PR	71490	7459	1019
3_1C	112160	14717	1328
3_1PB	109304	11956	1870
3_1PR	108210	12913	1915
3_2C	115822	18845	538
3_2PB	89214	6483	883
3_2PR	94059	8377	994
3_3C	95592	13020	444
3_3PB	283960	21464	3723
3_3PR	99024	7808	1303
3_4C	229929	16762	1944
3_4PB	119437	8168	1386
3_4PR	86083	7819	1097
Total	**8200048**	**1057018**	**69699**

Table S3.3: Microbial indicator and physico-chemical properties of influent and effluents of the unplanted and planted side of the rhizofiltration system. Values are the mean of triplicate samples and the standard deviation from the mean.

	Influent	**Unplanted**	**Planted**
FC log CFU.100 ml^{-1}	6.88 ± 3.85	6.31 ± 3.56	6.59 ± 3.80
SL log CFU.100 ml^{-1}	6.47 ± 3.59	5.76 ± 3.32	5.51 ± 3.15
NO_3^- mg.l^{-1}	0.02 ± 0.01	0.05 ± 0.03	0.02 ± 0.01
NH_4^+ mg.l^{-1}	71.27 ± 5.64	49.87 ± 4.78	46.97 ± 4.94
SO_4^{2-} mg.l^{-1}	20.78 ± 9.92	61.57 ± 25.77	62.47 ± 14.51
PO_4^{3-} mg.l^{-1}	10.32 ± 2.08	9.59 ± 2.80	11.84 ± 5.26
SS mg.l^{-1}	75.67 ± 3.28	37.00 ± 11.93	45.33 ± 12.45
COD mg.l^{-1}	393.67 ± 176.92	408.67 ± 179.72	382.00 ± 173.30
WT °C	20.00 ± 1.53	19.33 ± 1.20	19.00 ± 1
pH	6.5 ± 0.5	6.1 ± 0.6	6.1 ± 0.6

FC = faecal coliforms, SL = *Salmonella/Shigella,* SS = suspended solids, COD = chemical oxygen demand, WT= water temperature.

Supplementary material

Chapter 4

1 Yeast identification

The accuracy of the photographic identification guide to differentiate between yeast species, based on colony morphology on Molybdate Agar plates (Atlas, 1993), was confirmed by identifying random representatives of each morphological group using sequence analyses of taxonomic informative ribosomal gene sequences. Consequently, PCR amplification of the D1/D2 region of the 26S ribosomal RNA gene of these selected isolates was conducted using the primer set F63 (5'-GCATATACAATAAGCGGAGGAAAAG-3') and LR3 (5'- GGTCCGTGTTTCAAGACGG -3') (Inqaba Biotechnical Industries, Pretoria, RSA). The 25 µl PCR reaction mixture consisted of 10 µl KAPATaq Ready Mix (KAPA Biosystems, Cape Town, RSA), 1 µl of each primer (10 µmol/L), 3 µl of a single colony suspension in ddH20 and 10 µl ddH20. The PCR was performed in an Applied Biosystems 2720 Thermal Cycler (California, USA) with an initial denaturation temperature of 95 °C for seven minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds and elongation at 72 °C for one minute. The final elongation step was conducted at 72 °C for two minutes (Fell et al., 2000).

The PCR products were purified by means of a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals (Pty) Ltd., S.A.) and sequenced using a Hitachi 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA). Sequences were adjusted using Chromas Lite version 2.01 (Technelysium Pty. Ltd.). A search for highly similar DNA sequences in GenBank was performed using the Basic online Local Alignment Search Tool (BLAST) on the NCBI website. Sequences with ≥ 99 % homology to sequences obtained from our isolates were selected for further analysis. Additionally, the relevant sequences of type strains stored in the Centraalbureau voor Schimmelcultures (CBS), and NITE Biological Resource Center (NBRC) culture

collections, were obtained from GenBank. Sequences were aligned using the online multiple alignment program for amino acid or nucleotide sequences (MAFFT) version 6, (Computational Biology Research Center (CBRC) National Institute of Advanced Industrial Science and Technology (AIST), Japan) using the L-INS-i algorithm. The resulting alignments were viewed and re-aligned manually where necessary using the BioEdit V. 7 Sequence alignment editor. Subsequently, phylogenetic and molecular evolutionary analyses were conducted using Mega V. 5 (Tamura et al., 2011). A neighbour joining tree was constructed and the quality of the branching patterns was assessed by bootstrap resampling of the data sets with 1000 replications (Figure 1S). Sequences obtained in this study were submitted to GenBank.

Colony morphology, and the results of the abovementioned phylogenetic analyses, revealed the presence of seven different yeast species able to grow at 37°C (Figure 1S; Figure 2). The species were *Candida glabrata*, *Candida krusei, Candida tropicalis, Candida utilis, Clavispora lusitaniae (*anamorph *Candida lusitaniea) and Pichia rhodanensis*, as well as *Saccharomyces cerevisiae*.

2 Barcoded primers and DNA library preparation for high throughput sequencing

The modifications included the addition of a PGM sequencing adaptor, a “GAT” spacer and a unique error correcting Golay barcode. A set of 6 uniquely barcoded forward primers and one reverse primer was used for unidirectional multiplex sequencing (Table S4.1). PCR amplification was performed in a 15 µl reaction volume containing 7.5 µl 2x HiFi HotStart ReadyMix (Kapa Biosystems, Boston, MA, USA), 0.5 µM each of the forward and reverse primer and 50-200 ng DNA template. The PCR amplification conditions consisted of an initial denaturing step at 95 °C for 5 min followed by 35 cycles of 98 °C for 20 s, 65 °C for 15 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C

for 1 min and the samples were held at 4 °C. Amplicon product purification was performed via gel-electrophoresis using the e-gel system. Amplicon library concentration was estimated with the Qubit 2.0 instrument using the Qubit dsDNA HS assay (Life Technologies). The concentration of each sample was adjusted to 10-15 pM and all samples were pooled. This pooled sample was used for emulsion PCR according to the Ion PGM™ 200 Xpress™ Template Kit manual (Life Technologies, Carlsbad, USA).

3 References

Atlas, R.M., 1993. Handbook of Microbiological Media, in: Parks, L.C. (Ed.), CRC press, Boca Raton Florida, p. 614.

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G., Statzell-Tallman, A., 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evol. Microbiol. 50, 1351–1371.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Supplementary material

Chapter 4: Figures and tables

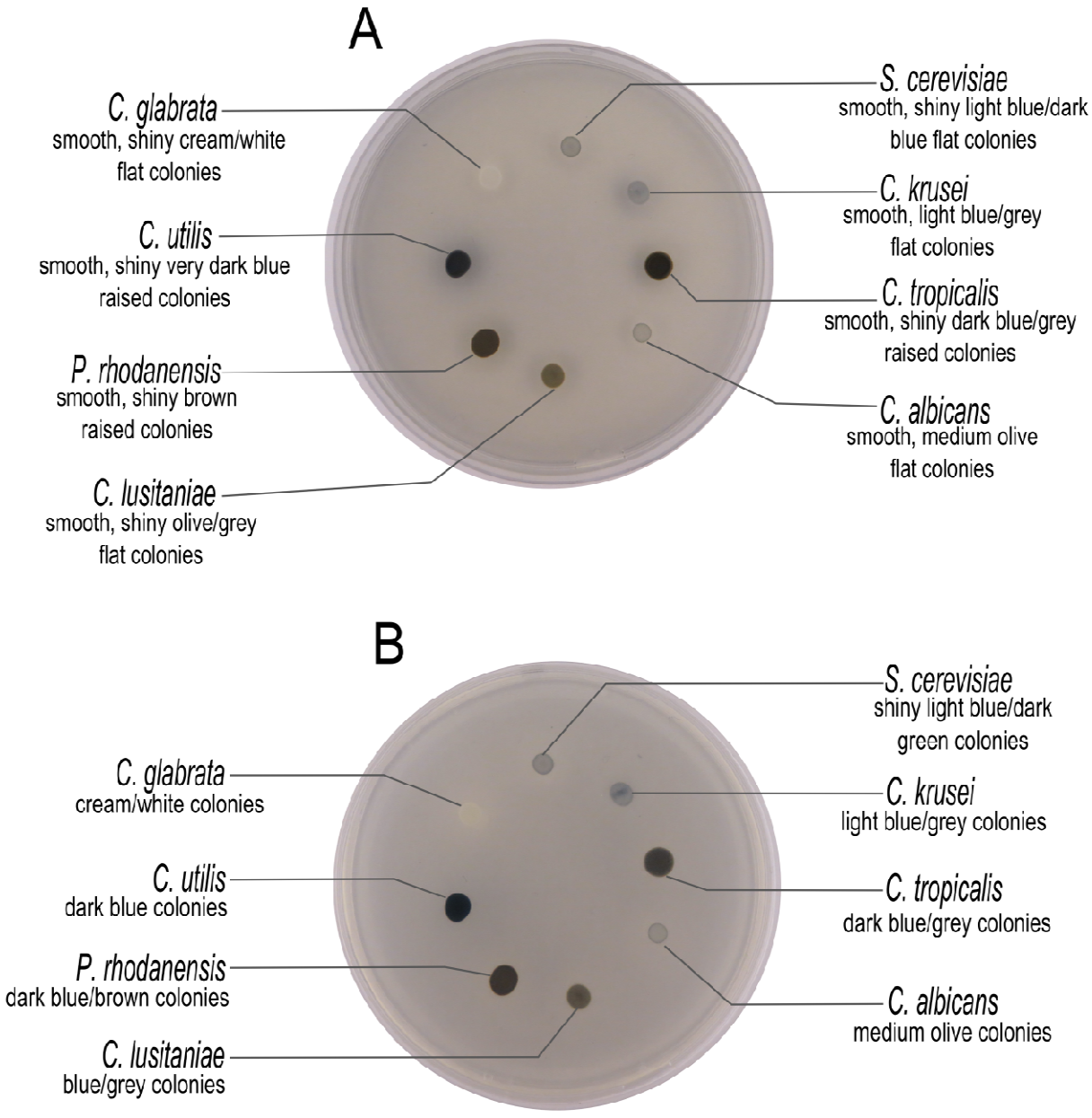

Figure S4.1: Colony morphology of potentially pathogenic yeast strains, representing different species, growing on molybdate agar. The photographs show the colonies from the top (A) and bottom (B) of the Petri dish. Strains: *Candida albicans* (CAB8908), *Candida glabrata* (CAB192), *Candida krusei* (CAB83), *Candida lusitaniae* (CAB76), *Candida tropicalis* (CAB632), *Candida utilis* (CAB78), *Pichia rhodanenis* (CAB77), and *Saccharomyces cerevisiae* (CAB618).

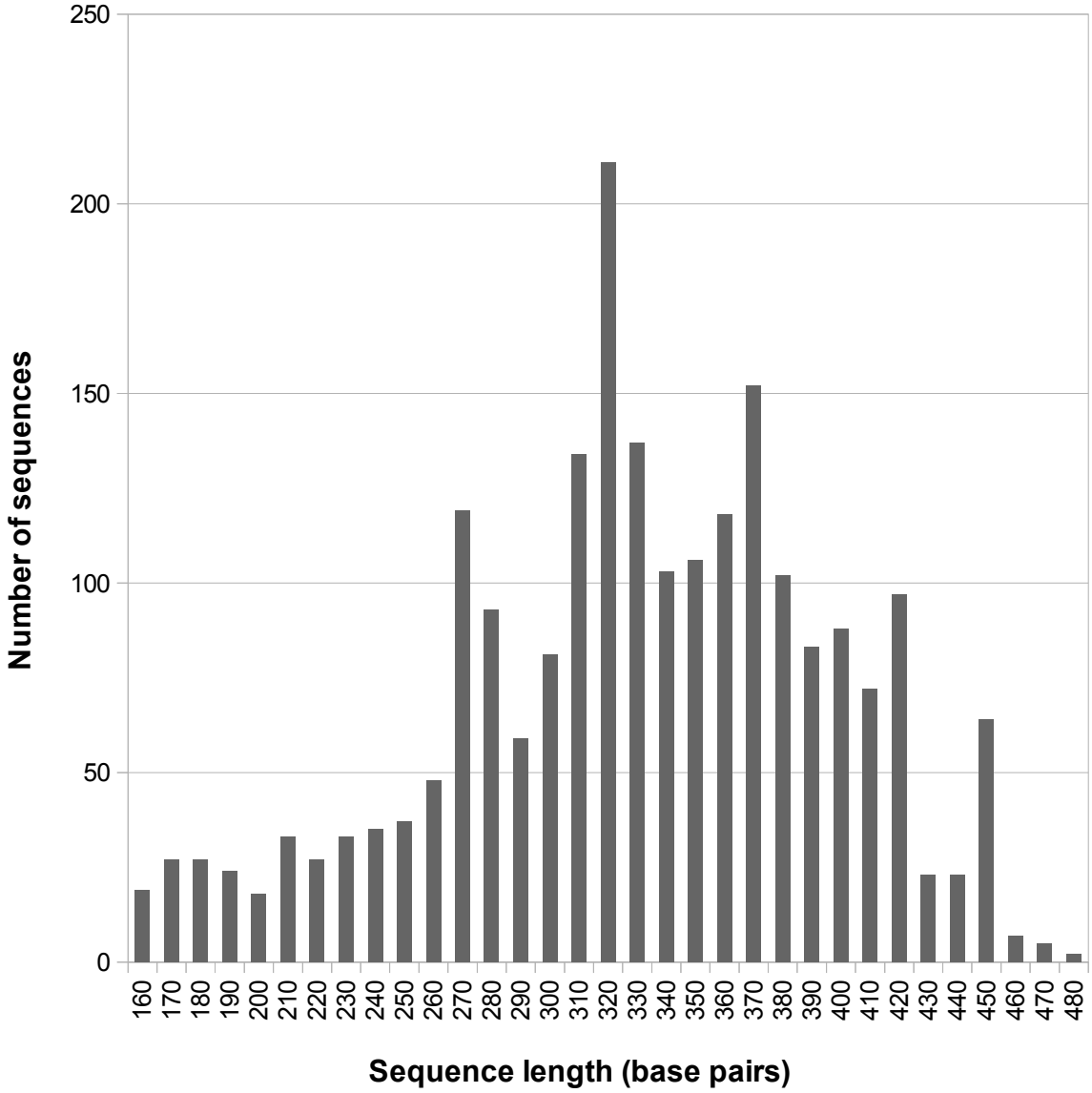

Figure S4.2: Sequence length distribution across the entire quality filtered sequence dataset.

Table S4.1: Barcoded primers sequences used for multiplex high throughput metagenome sequencing of the fungal ITS1 region using the IonTorrent personal genome machine.

Primer name	Adaptor sequence	Barcode	Barcode adaptor	Target Forward
ITSf_21	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	TCGCAATTAC	GAT	CT TGG TCA TTT AGA GGA AGT AA - 3'
ITSf_22	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	TTCGAGACGC	GAT	CT TGG TCA TTT AGA GGA AGT AA - 3'
ITSf_23	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	TGCCACGAAC	GAT	CT TGG TCA TTT AGA GGA AGT AA - 3'
ITSf_24	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	AACCTCATTC	GAT	CT TGG TCA TTT AGA GGA AGT AA - 3'
ITSf_25	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	CCTGAGATAC	GAT	CT TGG TCA TTT AGA GGA AGT AA - 3'
ITSf_26	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	TTACAACCTC	GAT	CT TGG TCA TTT AGA GGA AGT AA - 3'
				Target Reverse
ITS4R	5' - CCT CTC TAT GGG CAG TCG GTG AT			T CCT CCG CTT ATT GAT ATG C - 3'

Table S4.2: Number of sequences before and after quality control steps. C = unplanted control sand, P = planted sand, and time of sampling before (0 h) and after the addition of simulated urban effluent (1 and 168 h).

	Number of sequences						
	Total	**C0**	**C1**	**C168**	**P0**	**P1**	**P168**
Raw input	56391						
ITS subregion	41345						
Reads minus singletons and chimeras	36375	7883	4855	5967	5988	6108	5574